

## SURVEY AND SUMMARY

# Distribution and effects of amino acid changes in drug-resistant $\alpha$ and $\beta$ herpesviruses DNA polymerase

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### ABSTRACT

**Emergence of drug-resistance to all FDA-approved antiherpesvirus agents is an increasing concern in immunocompromised patients. Herpesvirus DNA polymerase (DNApol) is currently the target of nucleos(t)ide analogue-based therapy. Mutations in DNApol that confer resistance arose in immunocompromised patients infected with herpes simplex virus 1 (HSV-1) and human cytomegalovirus (HCMV), and to lesser extent in herpes simplex virus 2 (HSV-2), varicella zoster virus (VZV) and human herpesvirus 6 (HHV-6). In this review, we present distinct drug-resistant mutational profiles of herpesvirus DNApol. The impact of specific DNApol amino acid changes on drug-resistance is discussed. The pattern of genetic variability related to drug-resistance differs among the herpesviruses. Two mutational profiles appeared: one favoring amino acid changes in the Palm and Finger domains of DNApol (in  $\alpha$ -herpesviruses HSV-1, HSV-2 and VZV), and another with mutations preferentially in the 3'-5' exonuclease domain (in  $\beta$ -herpesvirus HCMV and HHV-6). The mutational profile was also related to the class of compound to which drug-resistance emerged.**

### INTRODUCTION

*Herpesviridae* are double-stranded linear DNA viruses that are responsible for multiple diseases in humans and present different tropism. The family is divided into  $\alpha$ ,  $\beta$  and  $\gamma$  subfamilies that contain eight human herpesviruses: herpes simplex virus 1 and 2 (HSV-1 and HSV-2), and varicella-zoster virus (VZV) (subfamily  $\alpha$ ); human cytomegalovirus

(HCMV), human herpesvirus 6 and 7 (HHV-6 and HHV-7) (subfamily  $\beta$ ); Epstein–Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV) (subfamily  $\gamma$ ). Their large linear genomes range from 125 to 235 kbp (Table 1) (1,2) and are protected by icosahedral capsids.

Herpesviruses are responsible for a wide variety of diseases and show neural and/or hematogenous dissemination. HSV-1 and HSV-2 are the causative agents of herpes *labialis* and herpes *genitalis*, respectively, as well as encephalitis and keratitis (3–7). VZV causes chickenpox and may reactivate to cause herpes zoster (mainly in elderly people). HCMV is responsible for infectious mononucleosis like syndrome, congenital cytomegalic inclusion body disease and a variety of manifestations in the transplanted recipient. These include CMV syndrome, graft infection, colitis, retinitis, pneumonitis and predisposition to opportunistic infections (8, 9). HHV-6 is the causative agent of *roseola infantum*, and encephalitis in rare cases (10). The  $\gamma$ -herpesviruses are oncogenic, since chronic EBV is linked to nasopharyngeal carcinoma and Hodgkin and non-Hodgkin lymphomas (Burkitt's lymphoma among others), besides infectious mononucleosis upon primary infection. KSHV is responsible for Kaposi's sarcoma (mostly in immunocompromised patients) as well as primary effusion lymphoma and multicentric Castelman's disease (11–13).

Since the discovery of the nucleoside analogue acyclovir in the 1970s by G. Elion, a new era in antiviral therapy based on nucleoside derivatives began (14,15). Several nucleoside analogues and their prodrugs have been designed and brought to the market: acyclovir (ACV, Zovirax®), ganciclovir (GCV, Cytovene®, Cymevene®), penciclovir (PCV, Denavir®, Vectavir®), their prodrugs [respectively, valacyclovir (Zelitrex®, Valtrex®), valganciclovir (Valcyte®), famciclovir (Famvir®)] and brivudine (Zonavir®, Zerpex®). Brivudine is different from the other

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**Table 1.** Human herpesviruses (HHV) are divided in 3 subfamilies ( $\alpha$ ,  $\beta$  and  $\gamma$ ). Genome size depends on the viral strain for all herpesviruses except HSV-1 and HSV-2

Subfamilies	Herpesvirus name	Acronym	Genome size ( $\approx$ kbp)
$\alpha$ -herpesvirinae	Herpes simplex virus 1	HHV-1/HSV-1	152.2
	Herpes simplex virus 2	HHV-2/HSV-2	154.7
	Varicella-zoster virus	HHV-3/VZV	124.8–125.4
$\beta$ -herpesvirinae	Human cytomegalovirus	HHV-5/HCMV	229.3–235.6
	Herpesvirus 6	HHV-6	159.3–162.1
	Herpesvirus 7	HHV-7	144.8–153.0
$\gamma$ -herpesvirinae	Epstein–Barr virus	HHV-4/EBV	171.8–172.7
	Kaposi's sarcoma associated herpesvirus	HHV-8/KSHV	137.5–165.0

antiviral agents since it has a proper sugar ring. Its originality is linked to the bromovinyl moiety present at the C5 position. Nucleoside analogues are the most frequently used antiherpesvirus drugs (Figure 1 and Table 2). To be active, nucleoside analogues necessitate activation to their triphosphate form in contrast to CDV and FOS (Figure 2). Foscarnet (FOS, Foscavir®), a pyrophosphate analogue, is commonly used as second-line treatment for drug-resistant herpesvirus strains (4–6,16–23). FOS directly targets the viral DNApol domain that interacts with the phosphate group of incoming nucleotides (24). Cidofovir (CDV, Vistide®), a nucleotide analogue of dCMP, is recommended only for multi-drug resistant strains with decreased sensitivity to nucleoside analogues and FOS. ACV, GCV, PCV and their prodrugs differ from natural nucleosides by the presence of hydroxyalkyl derivatives instead of a deoxyribose moiety. Similarly, CDV has no deoxyribose group but, in contrast to nucleoside analogues, harbors a phosphonate moiety.

Among the many genes expressed during viral replication, three of them are important in the therapy of herpesviruses: thymidine kinase (TK), protein kinase (PK) and DNA polymerase (DNApol). Herpesvirus TK or PK is necessary for the first phosphorylation step of nucleoside analogue activation. The two next phosphorylation steps are catalyzed by cellular kinases, leading to nucleoside triphosphate analogues that are efficient substrates of the herpesvirus DNApol. Incorporation of an activated analogue inhibits DNA polymerization, interrupting viral genome replication (Figure 2). Activation by the herpesvirus TK or PK is not required for CDV since it harbors a phosphonate moiety and therefore bypasses the first step of phosphorylation. FOS is a direct inhibitor of herpesvirus DNApol since it does not require activation.

Long-term exposure to antiviral drugs or suboptimal doses selects for mutations leading to drug-resistance. Emergence of drug-resistant herpesvirus strains has increased during the last decades, especially in the immunocompromised population (e.g. AIDS and organ and hematopoietic stem cell transplant patients). Commonly, single or multiple mutations appear in TK or PK, and to a lesser extent in DNApol. TK mutations result in lower drug activation, while DNApol mutations decrease the affinity of the polymerase for the triphosphate analogues and therefore result in weak inhibition of viral DNA synthesis. In recent years, CDV-(CDV<sup>R</sup>) and FOS-resistant (FOS<sup>R</sup>) strains have appeared and the mutant viruses have been genotyped, highlighting the urgent need for new antiviral agents with broad antiherpesvirus activity (25–29).

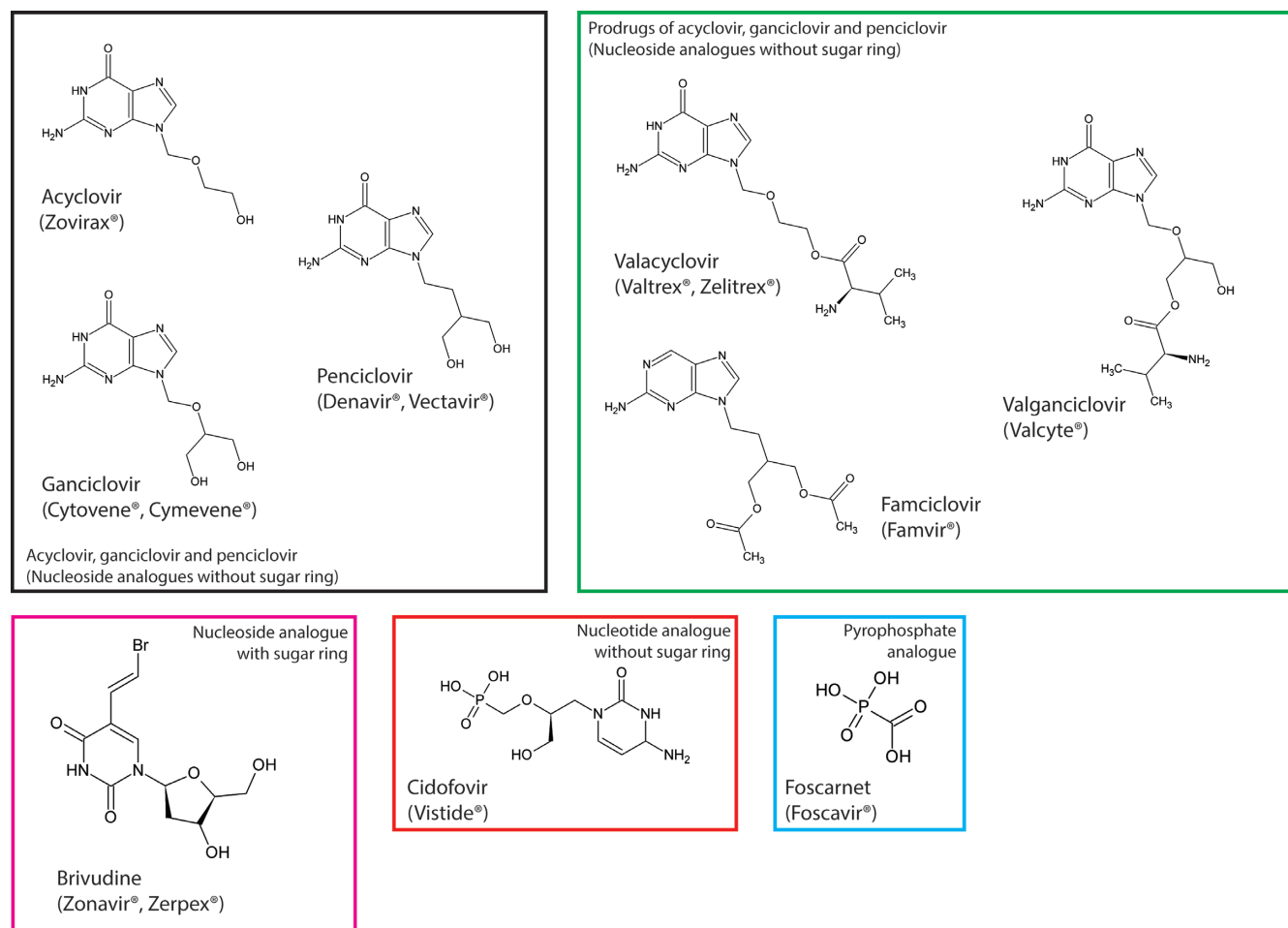
In this review, we focus on DNApol (Table 3) and the emergence of mutations after long-term treatment (for both clinical and laboratory strains) with antiherpesvirus nucleos(t)ide and pyrophosphate analogues used in antiviral therapy. The effects of these mutations on the three dimensional structure of the enzyme are discussed. In particular, we analyzed the distribution of mutations within the functional domains of DNApol for each herpesvirus, depending on the class of compounds used.

## STRUCTURAL FEATURES OF DNA POLYMERASE B FAMILY

### Functional domains of the type B DNA polymerases

DNApol of herpesviruses belongs to the type B family that comprises, among others, the human DNApol  $\alpha$ ,  $\delta$ ,  $\epsilon$  and  $\zeta$  and the *Enterobacteria* phage RB69. Type B DNA polymerases are composed of functional domains designated the N-terminal domain, the Finger/Palm/Thumb domains and the 3'-5' exonuclease domain (responsible for the proof-reading activity) (Figure 3). These domains work together to produce high fidelity replication of the genome. Residues in the Palm and Finger domains are involved in catalysis and binding of incoming nucleoside triphosphates. The thumb domain interacts with the primer–template complex. The architecture of the type B DNApol harbors a 3'-5' exonuclease domain whose role is to correct misincorporated nucleotides and to maintain the fidelity and integrity of the newly formed DNA molecules (30,31). Interestingly, the HSV-1 DNApol has an extra domain, the pre-NH<sub>2</sub>-terminal domain, according to the three dimensional structure published by Liu *et al* (32). This domain is required for efficient viral replication as well as for establishment of latency (as observed experimentally in mice) (33,34). In EBV DNApol, the pre-NH<sub>2</sub>-terminal domain is also important for lytic genome replication (35).

The bacteriophage RB69 DNApol is one of the most studied at the structural and functional levels, and there are currently 122 entries in the protein data bank (<http://www.rcsb.org/pdb/results/results.do?outformat=&qrid=C9789076&tabtoShow=Current>) (30,36–40). Although RB69 DNApol lacks the pre-NH<sub>2</sub>-terminal domain, it is a good surrogate model for herpesvirus DNApol, especially regarding structural changes involved in catalysis and ligand binding (DNA, dNTPs) (36). HSV-1 DNApol structure is also a good structural model for the other HHVs since the sequence identity is high among



**Figure 1.** Structure of the most common nucleos(t)ide analogues used in antiherpesvirus therapy. The antiherpesvirus pharmacopeia includes acyclovir, ganciclovir, penciclovir and their prodrug products valacyclovir, valganciclovir and famciclovir, as well as brivudine, cidofovir and foscavir.

**Table 2.** Antiherpesvirus agents and their clinical applications

Antiherpesvirus agents	Trade names	Indication	Route of administration	References
Acyclovir	Zovirax®	HSV-1, HSV-2, VZV	Oral, i.v., topical	(4–6,14,15,19)
Valacyclovir	Valtrex®, Zelitrex®	HSV-1, HSV-2, VZV	Oral	(5,6,14,15,21)
Ganciclovir	Cytovene®, Cymevene®	HCMV, HHV-6*	Oral, i.v., intravitreal	(16,17)
Valganciclovir	Valcyte®	HCMV, EBV*, HHV-6*	Oral	(16,17)
Penciclovir	Denavir®, Vectavir®	HSV-1, HSV-2, VZV	Topical	(18,21,23)
Famciclovir	Famvir®	HSV-1, HSV-2, VZV	Oral	(18,23)
Brivudine	Zostex®, Zerpex®	HSV-1, VZV	Oral	(18,20)
Cidofovir	Vistide®	HCMV, HSV-1, HSV-2	i.v.	(14,17,18)
Foscarnet	Foscavir®	HCMV, HSV-1, HSV-2, HHV-6*	i.v.	(14,17)

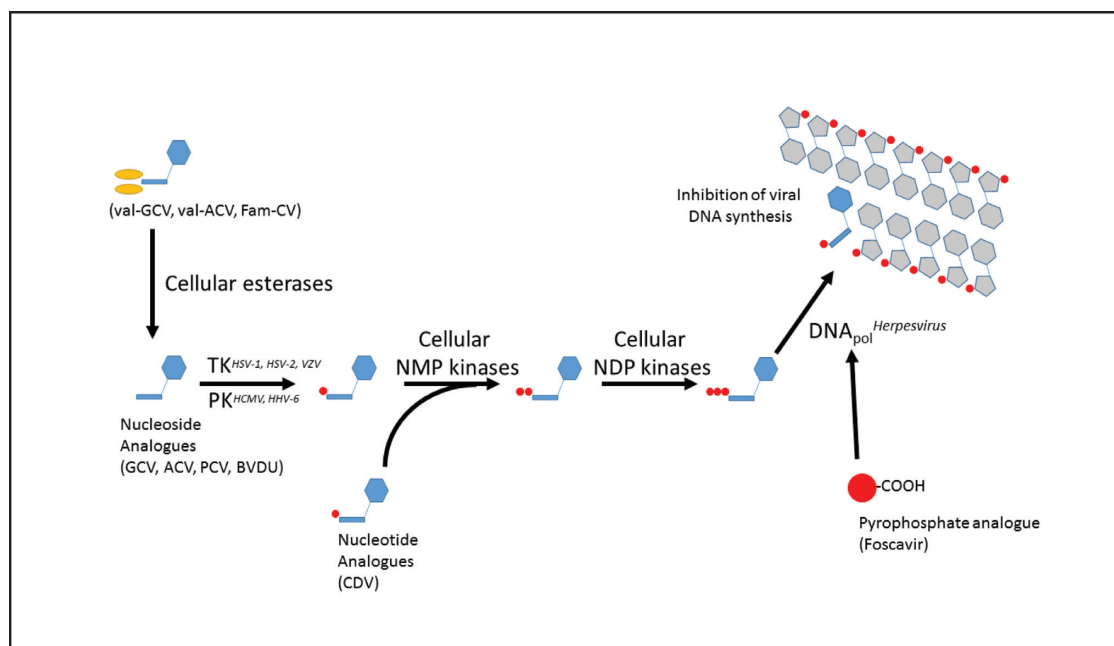
(i.v.: intravenously; \*: efficacy under investigation).

the members of the herpesviridae resulting in conserved protein-folding (32).

### Catalytic features responsible for the polymerization activity

The interface between the Finger and Palm domains is important for the catalytic activity of DNApol. Two aspartates in RB69 DNApol, D411 and D623, establish a network of hydrogen bonds with the  $\alpha$  and  $\gamma$  phosphates of the incom-

ing nucleoside triphosphate, directly or via magnesium ions (Figure 4A; active site of RB69 DNApol with incoming dCTP). In a similar manner, polar residues from the Finger domain also interact with the three phosphate moieties of dCTP and the 3'-hydroxyl group of dCTP deoxyribose. These interactions involve R482, K486, K560 and N564. It is worth noting that an aromatic residue, Y416, reinforces the stability of the incoming nucleotide via stacking inter-



**Figure 2.** Antiviral strategy for inhibition of viral replication by targeting viral DNApol. Nucleoside analogues are active following three step of phosphorylation catalyzed by the viral thymidine kinase (TK) and protein kinase (PK), base specific cellular nucleoside monophosphate kinases, and nucleoside diphosphate kinases. The active triphosphate form is recognized and incorporated in the newly synthesized viral DNA by the herpesvirus DNApol that inhibits viral replication. The first phosphorylation step is the bottleneck in activating nucleoside analogues. Prodrugs of ACV, GCV and PCV were designed to increase their bioavailability. Cellular esterases release the nucleoside form following drug uptake. It is worth noting that HSV-1 TK and VZV TK perform two phosphorylation steps to activate BVDU, forming both BVDU-MP and BVDU-DP.

**Table 3.** The type B DNApol family includes *Herpesviridae* DNApol, human DNApol  $\alpha$ ,  $\delta$  and  $\epsilon$  and the *Enterobacteria* phage RB69 protein

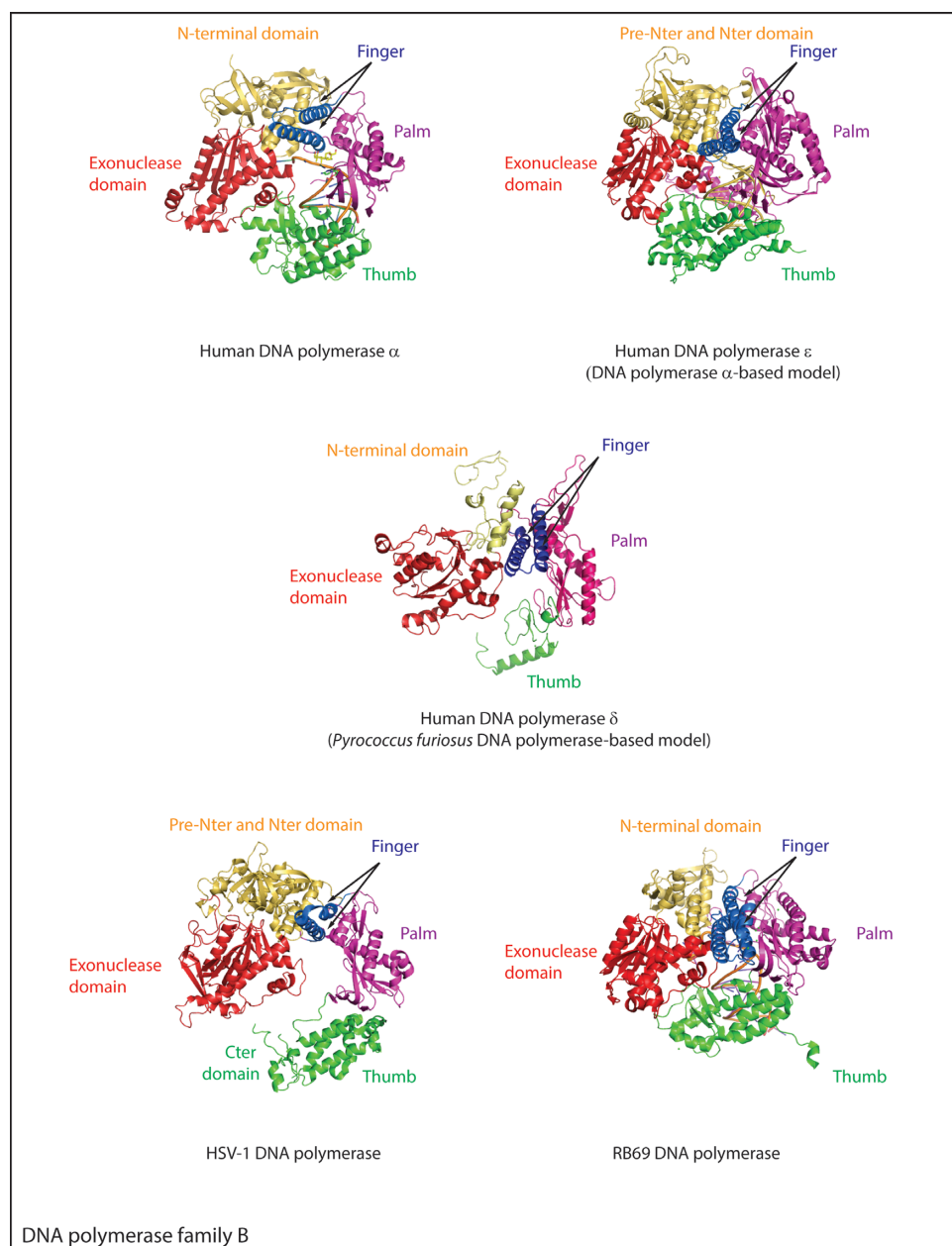
Formal Name	Common name	Gene	UniProt entry	Length (amino acids)
Human Herpesvirus 1 (HHV-1)	Herpes simplex 1 virus (HSV-1)	UL30	B2ZUN5	1235
Human Herpesvirus 2 (HHV-2)	Herpes simplex 2 virus (HSV-2)	UL30	P89453	1240
Human Herpesvirus 3 (HHV-3)	VZV	ORF28	Q997N0	1194
Human Herpesvirus 4 (HHV-4)	EBV	BALF5	P03198	1015
Human Herpesvirus 5 (HHV-5)	HCMV	UL54	P08546	1242
Human Herpesvirus 6 (HHV-6)	-	U38	P28857	1012
Human Herpesvirus 7 (HHV-7)	-	U38	P52342	1012
Human Herpesvirus 8 (HHV-8)	Kaposi's sarcoma associated herpesvirus (KSHV)	ORF9	Q2HRD0	1012

actions between its side chain and the sugar ring of the nucleotide. A comparison can be made with the active site of HSV-1 UL30 whose 3D-structure has been published in the apo enzyme form (no substrate). Figure 4B represents the active site of HSV-1 DNApol in the open conformation without incoming nucleotide. There is a high degree of sequence identity between residues K786, R789, K811 and N815 in HSV-1 DNApol and the homologous positions of R482, K486, K560 and N564 in RB69 DNApol Finger domain. Catalytic residues D411 and D623, as well as Y416 are important for dCTP binding in RB69 DNApol (Figure 4A). These residues are conserved in HSV-1 DNApol (homologous positions D717, D888 and Y722). To be a functional active site, the Finger domain in HSV-1 DNApol undergoes an important movement in order to adapt the environment for the incoming dNTP and the template DNA. Residues in the Finger and Palm domains move closer to stabilize the complex with the DNA molecule and the dNTP. Interestingly, among these important residues shaping

the active site of HSV-1 DNApol, only N815 was mutated in drug-resistant HSV-1 strains in patients. The other residues (K786, R789 and K811) were not affected by mutations (Supplementary Table S1). Indeed, none of the homologous residues were mutated in other drug-resistant herpesviruses, including HSV-2, HCMV and HHV-6.

On the contrary, the homologous position N815 is also affected in VZV DNApol (i.e. N779S), that conferred ACV<sup>R</sup> (41). N815 establishes interactions with the 3' OH moiety of the incoming dNTP, which is a critical recognition step for dNTP incorporation. N815 alterations may affect the recognition of ACV-TP because it lacks a proper deoxyribose group. In HSV-1 DNApol, several amino acid changes were identified at position 815 with different physicochemical properties (apolar residues leucine and valine, polar residues tyrosine, glutamate and serine), confirming the importance of an asparagine residue at this position. Amino acids other than asparagine could affect the sensitivity to nucleoside analogues by modifying the chemical en-





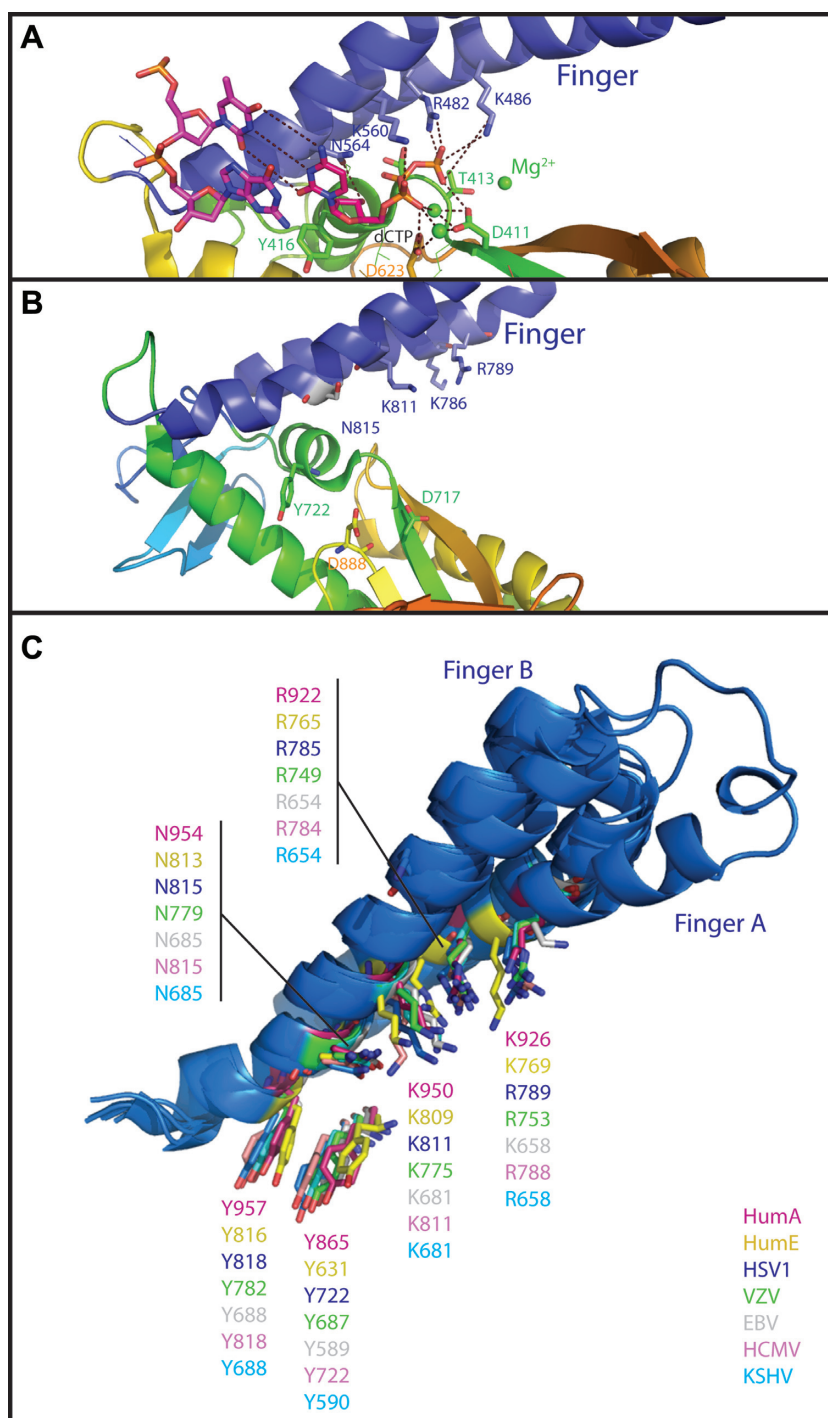
**Figure 3.** Three dimensional structures of human DNAPol  $\alpha$ ,  $\delta$  and  $\epsilon$ , HSV-1 DNAPol and RB69 DNAPol. The ternary structure is very similar, with conserved functional domains in the N-terminus (yellow), Finger (blue), Palm and Thumb (purple and green, respectively) and the Exonuclease (red). Herpesviruses DNAPol possess a pre-N-terminus domain that is not well studied. For human DNAPol  $\delta$  and  $\epsilon$ , the model of the 3D-structure was built using 'Swiss-Model workspace' (<http://swissmodel.expasy.org/>). All the structures were visualized and the pictures generated using PyMol Delano Software.

vironment around position 815 (i.e. shorter side chain, negatively charged residue or hydrophobic amino acid).

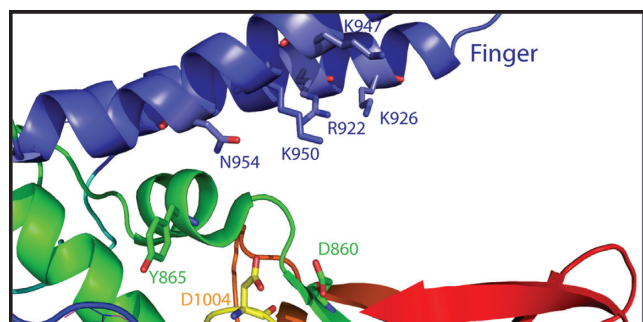
### Conservation of the finger domain among the type B family DNA polymerases

The Finger domain is highly conserved in the type B DNAPol family. Figure 4C is a superimposition of several DNAPol Finger domains (human  $\alpha$  and  $\epsilon$ , HSV-1, VZV, EBV, HCMV and KSHV) showing key residues in the domain structure and function. The DNAPol  $\alpha$  positions Y865, R922, K926, K950, N954 and Y957 are con-

served (aa865, aa922, aa950, aa954 and aa957) or similar (aa926) in herpesvirus DNAPol or in human DNAPol  $\epsilon$ , and shape the three dimensional structure of the Finger domain. Their polar and/or positively charged side chains allow establishment of electrostatic or H-bond interactions with the incoming dNTPs. This domain is critical for catalysis of the polymerase reaction, and the Finger A and Finger B helices are subject to an important rotation to adopt an optimal position for substrate recognition (36). A close view of the human DNAPol  $\alpha$  active site shows the same arrangement as the RB69 and HSV-1 DNAPols [pdb: 4Q5V; (42)] (Figure 5). The important residues for catalysis in the



**Figure 4.** (A) RB69 DNAPol active site complexed to an incoming dCTP. (B) HSV-1 DNAPol active site in an open configuration without incoming nucleotide. (C) Comparison of the Finger domain from human DNAPol  $\alpha$  (pdb: 4Q5V) and  $\epsilon$  (model), HSV1 DNAPol (pdb: 2VG9), VZV DNAPol (model), EBV DNAPol (model), HCMV DNAPol (model) and KSHV DNAPol (model). Critical amino acids that bind nucleoside triphosphates are shown in stick representation. Key residues are conserved except at position K926 (in human DNAPol  $\alpha$ ). A lysine (K) is present at this position in human ( $\alpha$  and  $\epsilon$ ), and an arginine is found at this position in EBV, HSV-1, VZV, HCMV and KSHV DNAPol. The green spheres represent calcium ions (A). All the structures were visualized and the pictures generated using PyMol Delano Software.



**Figure 5.** DNA template binding in the active site of human DNAPol  $\alpha$ . The Finger (blue) and the Palm domains (green) form the active site to interact via polar or aromatic residues with the incoming nucleotide. Pymol Delano software was used to visualize and generate this figure.

Finger domain of DNAPol  $\alpha$  are R922, K926, K947, K950 and N954 as well as the dyptich D860/D1004 (homologous residues of D411/D623 in RB69 and D717/D888 in HSV-1). Since the Finger domain interacts closely with dNTPs and therefore with antiherpesvirus drugs, it has been identified as a preferential site for mutations that confer resistance. Structural similarities among DNAPol B type family members allow extrapolation to herpesvirus DNAPols, at least for the catalytic process of DNA polymerization, and to obtain insights into the effects of mutations in drug-resistant herpesviruses.

### Selectivity of antiherpesvirus agents toward the viral DNA polymerase

*Distinct modes of action among the antiherpesvirus agents.* The antiherpesvirus agents presented in this review have a variety of mechanisms of action leading to the inhibition of viral DNA replication. The various mechanisms of action are due to the chemical and structural heterogeneity of these drugs, which interact in different ways with the viral DNAPol (Figure 6). ACV triphosphate (ACV-TP) competitively inhibits dGTP binding and is incorporated into DNA by HSV-1/-2 and VZV DNAPol to act as a chain terminator (Figure 6A). ACV incorporated at the 3' extremity of the elongated DNA is also considered a suicide inhibitor because the viral DNAPol cannot bypass its presence through the 3'-5' exonuclease activity (43,44). Although GCV triphosphate (GCV-TP) is structurally related to ACV-TP, its mode of action is slightly different (Figure 6A). GCV-TP is both a competitive inhibitor and a substrate of HCMV DNAPol. It is incorporated into viral DNA but is not considered an obligate chain terminator. Viral DNA elongation can still be catalyzed, but the process stops after one additional nucleotide is incorporated. Like GCV-TP, PCV triphosphate (PCV-TP) is a competitive inhibitor of the HSV/VZV DNAPol and it is a substrate that allows incorporation of an additional nucleoside (Figure 6A). The mechanism of action of brivudin distinguishes it from ACV, GCV and PCV (Figure 6B). The triphosphate form (BVDU-TP) is a substrate of VZV DNAPol and is a competitive inhibitor with respect to dTTP. However, its incorporation does not stop elongation of viral DNA but alters the DNA structure leading to functionally disabled

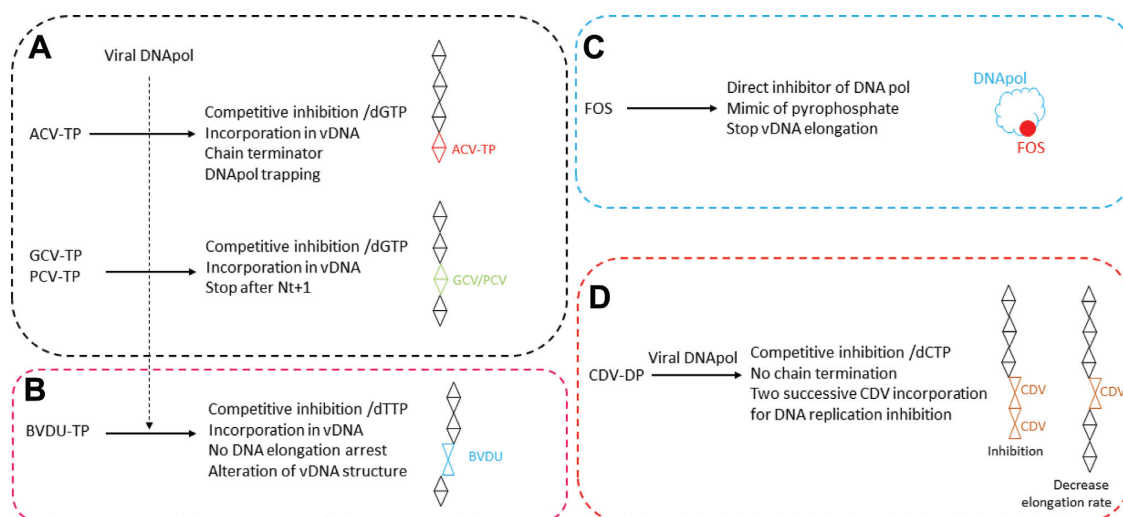
viral genomes (45,46). Foscavir (FOS) directly targets the dNTP binding domain of viral DNAPol by mimicking pyrophosphate and preventing efficient binding of the incoming dNTP (Figure 6C). It interacts with residues that recognize the  $\beta$  and  $\gamma$  phosphates of the incoming nucleotide (47). It impacts viral DNA elongation by aborting the process. Cidofovir diphosphate (CDV-DP) is a competitive inhibitor with respect to dCTP and is also a substrate of the viral DNAPol (Figure 6D). No chain termination is observed unless two successive molecules of CDV-DP are incorporated into the viral genome. If only one molecule of CDV-DP is incorporated, then DNA replication continues at a significantly decreased elongation rate (48).

*Inhibitory effects of nucleoside triphosphate analogues on viral DNA polymerases.* The triphosphate forms of antiherpesvirus nucleoside analogs are competitive inhibitors of viral DNAPol with respect to the natural nucleoside triphosphates dCTP, dGTP and dTTP. The effects of these antiviral triphosphates were investigated using viral (HSV-1, HSV-2, VZV and HCMV) and human DNAPol  $\alpha$  in enzyme assays (Table 4) (21,46,49–61). The kinetic parameters were measured, although values differed depending on the types of assays used. Sources of variation could be attributed to the origin of the proteins (extracts of infected cells, recombinant proteins expressed from baculovirus, etc.) and the level of purity.

The inhibitory constants ( $K_i$ ) of ACV-TP for HSV-1 UL30, HSV-2 UL30, VZV ORF28 and HCMV UL54 were in the nanomolar range. When tested with human DNAPol  $\alpha$ , the  $K_i$  values were in the submicromolar/micromolar range (0.1–3.8  $\mu$ M). The selectivity ( $K_i$  human DNAPol  $\alpha$  divided by  $K_i$  viral enzyme) for viral DNAPol (HSV-1/2, HCMV) ranged between 12 and 132 (52,53,56). A  $K_i$  value of 990 nM was measured with HSV-1 UL30, but human DNAPol  $\alpha$  was not tested in this study and so the selectivity ratio was not calculated (54). Similar  $K_i$  values were obtained with GCV-TP when tested with HSV-1, HSV-2 and HCMV DNAPol, and the selectivity ratio ranged from 4 to 7.3 due to greater inhibition of human DNAPol  $\alpha$  (53,56,62,63).

PCV-TP was tested for inhibition of HSV-1, HSV-2 and VZV DNAPol (21, 58, 64). When activated by phosphorylation, the (*S*)-enantiomeric form is predominantly produced (>95%) in HSV-infected cells. The inhibitory constants were in the micromolar range (1.6–11  $\mu$ M) while a  $K_i$  of 175  $\mu$ M was measured for DNAPol  $\alpha$  (21). Earnshaw *et al.* found that the inhibitory effect of PCV-TP on HSV-1 and HSV-2 DNAPol activity was 82–121 times weaker than that of ACV-TP ( $K_i$  around 70 nM) (21).

BVDU triphosphate (BVDU-TP) inhibitory activity was assessed on HSV-1, HSV-2, VZV and HCMV DNAPol and compared to human DNAPol  $\alpha$  (50,51,57,61). Its effect on DNAPol  $\alpha$  was in the range of 0.7–3.6  $\mu$ M. The  $K_i$  of BVDU-TP for HSV-1 and HSV-2 DNAPol was 54–250 nM, while it was 550 nM for VZV DNAPol. It is worth noting that BVDU is inactive against HSV-2 despite similar  $K_i$  values of BVDU-TP for HSV-1 and -2 DNAPol. The reason is that in HSV-2 infected cells viral TK does not convert BVDU-MP to BVDU-DP (through TMP kinase-associated activity) (65–67). BVDU-TP was less potent



**Figure 6.** Modes of action of the different antiherpesvirus agents presented in this commentary. The triangles represent nucleotides forming the viral DNA chain.

**Table 4.** Kinetic parameters of natural nucleotides and antiherpesvirus agents for HSV-1, HSV-2, VZV and HCMV DNApol and human Pol  $\alpha$ .  $K_M$  represents the concentration of substrate required for 50% enzyme activity, while  $K_I$  represents the concentration of active nucleoside/nucleotide analogue required to inhibit DNApol activity by 50%. These constants reflect binding of the inhibitor to the enzyme. References used to build this Table are in parentheses.  $K_I$  was not available in some cases, but the  $IC_{50}$  of the drug was published. The (*S*)- or (*R,S*)-enantiomeric forms of penciclovir triphosphate were used in these different studies to measure inhibitory constant values

DNA polymerases	K <sub>M</sub> (nM)			K <sub>I</sub> (nM)					
	dGTP	dCTP	dTTP	ACV-TP	GCV-TP	PCV-TP	BVDU-TP	Foscavir	CDV-DP
HSV-1 UL30	140 (54)	310 (68)	140 (57)	3 (52,53)	30 (61)	3700 (58)	68 (57)	40 (76)	860 (68)
	150 (52)	500 (61)	500 (61)	5.1 (58)	33 (53)	85 000 (21)	250 (50)	400 (79)	
	200 (61)		660 (50)	25 (59)		11 000 (64)	IC <sub>50</sub> = 750 (51)	600 (73)	
	235 (53)			70 (21)				IC <sub>50</sub> = 3000 (72)	
	570 (21)			990 (54)					
HSV-2 UL30	140 (53)	100 (61)	180 (57)	3 (52,53)	46 (53)	3000 (58)	54 (57)	16 000 (75)	1400 (68)
	150 (52)	370 (68)	200 (61)	6.7 (58)		6000 (64)	68 (61)		
	200 (61)		850 (55)	70 (21)		5800 (21)			
	360 (21)			80 (64)					
VZV ORF28	640 (49)	-	1430 (46)	5.3 (58)	-	1600 (64)	550 (46)	IC <sub>50</sub> = 1000 (49)	-
	1300			10 (64)		3600 (58)	IC <sub>50</sub> = 490 (51)	IC <sub>50</sub> = 180 (51)	
HCMV UL54						IC <sub>50</sub> = 37 500 (21)			
	110 (78)	100 (78)	310 (78)	8 (56)	22 (56)	-	IC <sub>50</sub> = 3600 (51)	300 (78)	1200 (62)
	240 (62)	230/320 (63)	550 (56)		70/90 (63)			350/450 (63)	6600 (69)
	160/260 (63)	260 (62)	600 (61)		85 (62)			400 (80)	
	440 (105)	570 (56)	932 (74)		4500 (105)			550 (76)	
	470 (56)	600 (61)						1250 (74)	
	500 (61)	884 (74)						3500 (62)	
POLα								IC <sub>50</sub> = 9800 (51)	
	970 (21)	1450 (56)	1200 (57)	96 (56)	80 (60)	175 000 (21)	700 (57)	IC <sub>50</sub> > 20 000 (194)	51 000 (68)
	1200 (52)	4700 (68)	1540 (56)	180 (52)	120 (61)		3600 (50)	IC <sub>50</sub> = 7700 (51)	
	1430 (56)		5300 (50)	200 (61)	146 (56)		3600 (61)		
				3300 (59)				IC <sub>50</sub> = 21 000 (51)	
			3800 (21)						

when tested with HCMV DNApol, with an  $IC_{50}$  of 3.6  $\mu$ M (51).  $K_M$  and  $K_I$  values for dTTP and BVDU-TP for EBV DNApol were 13  $\mu$ M and 16.1  $\mu$ M, respectively (50).

*Inhibitory effects of the nucleotide analogue cidofovir and pyrophosphate analogue foscavir on viral DNA polymerases.* The inhibitory effects of CDV-DP on viral DNApol were investigated for HSV-1 and HSV-2 UL30 as well as for HCMV UL54 (Table 4) (62,63,68,69). The inhibitory constants were in the range of 0.86 to 6.6  $\mu$ M (for the viral enzymes) while the  $K_I$  for the human DNApol  $\alpha$  was about 51  $\mu$ M (68,69). The selectivity for viral DNApol was between 36 and 59. The structure of an oligonucleotide duplex containing CDV was solved using NMR (70). Incorporation of

CDV was well tolerated and did not dramatically alter the DNA configuration, which was a common B-type. However, the stability of the duplex was weaker than a duplex without incorporated CDV. In contrast, the DNA conformation of an oligonucleotide duplex bearing a ganciclovir molecule was more altered, switching from the classical B-type to the A-type nucleic acid that is typical of RNA/RNA or RNA/DNA duplexes (71).

The inhibitory effects of FOS were investigated for HSV-1/HSV-2 UL30, VZV ORF28 and HCMV UL54 (Table 4) (51,62,63,72–80).  $K_I$  values ranging from 40 to 600 nM were measured for HSV-1 UL30 DNApol. In Abele *et al.*, the  $IC_{50}$  of FOS for VZV DNApol was measured below 1  $\mu$ M (49) while in another study, an  $IC_{50}$  of 180 nM was observed



(51). In contrast,  $K_I$  values ranging from 300 nM to 3.5  $\mu$ M were obtained with HCMV UL54 DNApol (62,74,76,78). This difference in inhibitory effects might be due to the assay used, its sensitivity or to the quality of the protein purification. When tested on cellular DNApol  $\alpha$ ,  $IC_{50}$  values for FOS ranged from 7.7 to 20  $\mu$ M (51).

**Structure-activity relationship of the selectivity toward viral DNA polymerases.** Most antiherpesvirus agents exhibit a preference for viral DNApol, with  $K_I$  values in the nanomolar range compared to the micromolar range for human DNApol  $\alpha$  (Table 4). This preference is also reflected at the level of the natural NTP (e.g. dCTP, dGTP and dTTP), supporting the hypothesis that the active site of viral DNApol harbors few amino acid variations that contribute to substrate selectivity. Figure 7 shows several structural features that differ between human DNApol  $\alpha$  and HSV-1 UL30. Three distinct residues were identified at positions N862, K926 and K946 in human DNApol  $\alpha$  and A719, R789 and A808 in HSV-1 UL30 (Figure 7A). Residues K926 and K947 in human DNApol (R789/A808 in HSV-1) interact directly with the incoming nucleoside triphosphate (i.e. dCTP in Figure 7B and C). The amino acid variations in HSV DNApol (Lys to Arg/Ala) may greatly modify substrate binding by increasing the side chain volume [Lys (171Å) and Arg (225Å)] or the hydrophobicity of the residue [percent of buried residue Lys (4.2%) and Ala (38%)] (Figure 7B and C). This suggests better interactions with the incoming dNTP or a better accommodation of the substrate in the pocket of the active site. The accommodation of nucleoside and nucleotide analogues with an acyclic part (instead of a sugar ring) might be favored near residues that form a wider active site. Ala-719 in HSV-1 is conserved in all herpesviruses except HHV-6 and HHV-7 (Gln574/Gln573). However, the kinetic parameters of DNApol have not been studied for these  $\beta$ -herpesviruses, and so we cannot draw conclusions on the impact of the Ala to Gln variation on substrate selectivity.

FOS binds the DNApol active site differently than nucleoside and nucleotide analogues. It competes with the  $\beta$  and  $\gamma$  phosphates of the dNTP, establishing interactions with polar residues from the Finger domain (Asn, Arg and Lys), as well as with the catalytic aspartates (Figure 7D). Zahn *et al.* solved the structure of RB69 DNApol in complex with foscavir and acycloGMP (pdb code 3KD5) (47). Foscavir binds the active site through two groups of interactions. On one hand, R482, K486, K560 and N564 interact with foscavir through direct hydrogen-bonds or via water molecules. On the other hand, the catalytic aspartates D411 and D623 bridge the foscavir molecule via a magnesium ion (Figure 7D).

## AMINO ACID CHANGES CONFERRING DRUG-RESISTANCE PHENOTYPE

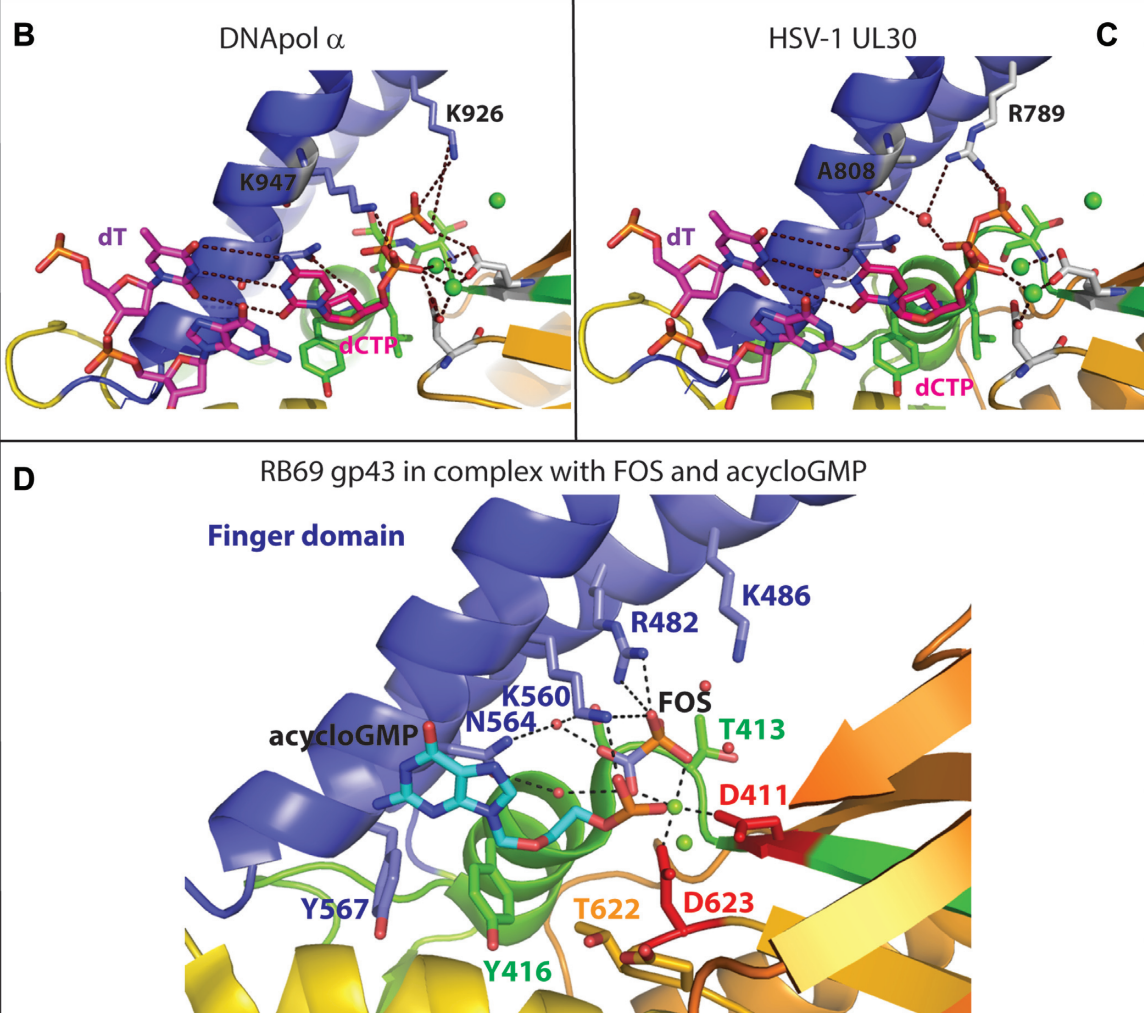
A challenging problem in the treatment of herpesvirus infections in immunocompromised patient populations is to overcome the viruses harboring mutations in TK, PK and DNApol genes (24). The genetic evolution of the virus aims to bypass the effects of the drug in order to replicate the viral genome. The mutations have a variable impact on the en-

zyme and, in the case of DNApol, the mutated position can affect different domains causing different activities (polymerase, 3'-5' exonuclease). Thus, understanding the distribution of mutations in herpesviruses DNApol is critical not only for insight into amino acid changes in the polypeptide, but also for the design of new antiherpetic agents that bypass these mutations.

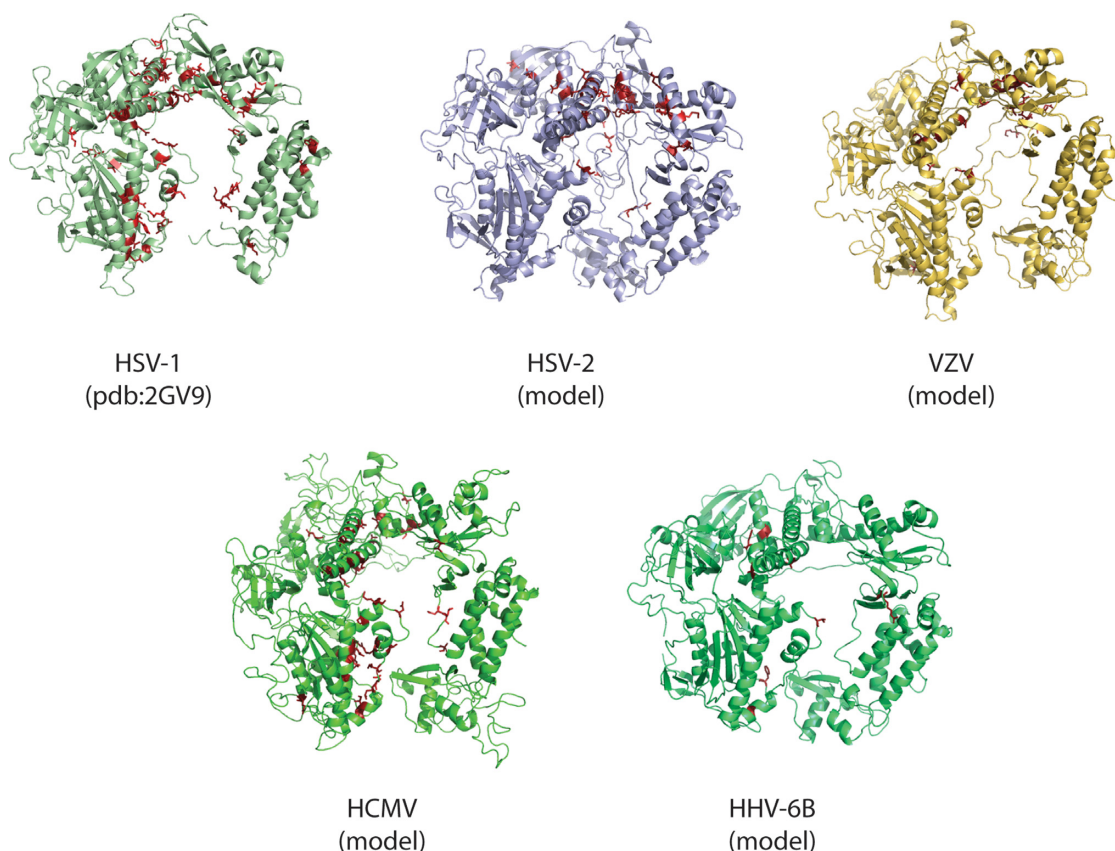
## Distribution of mutations in the herpesviridae DNA polymerase polypeptide

**Effects of mutation distribution on the function of DNApol domains.** Mutations conferring drug-resistance to a wide family of antiherpesvirus agents have been described in the last decades (81–88) (Supplementary Data, Table 1). These include mutations identified in patient samples as well as in laboratory strains selected under drug-pressure. Among the eight herpesviruses, data are available for HSV-1, HSV-2, VZV, HCMV and HHV-6 (Figure 8). In 2009, our laboratory initiated the RegaVir translational platform to genotype and phenotype herpesvirus strains from patients with therapeutic failure. This project aimed to advise clinicians on the therapeutic strategy to treat patients that do not respond to antiviral therapy, and to identify new mutations leading to drug-resistance. Here, we analyzed the distribution of mutations per enzyme domain based on amino acid changes described in the literature or identified by the RegaVir platform. Figure 9 summarizes the distribution of mutations identified per domain in each herpesvirus. Two distinct patterns emerged: one group composed of HSV-1, HSV-2 and VZV with most mutations (69–89%) in the Palm/Finger/Thumb domains, and a second group composed of HCMV and HHV6A with most mutations (60–75%) in the 3'-5' exonuclease (Figure 9). Identification of additional drug-resistance mutations in HHV6A beyond the eight identified so far will increase the accuracy of the mutation profile.

Models of the DNApol of HSV-2, VZV, HCMV and HHV-6 were built on the basis of HSV-1 DNApol structure in order to analyze the distribution of the identified mutations (32). A comparison of the Palm and 3'-5' exonuclease domains of HSV-1 and of a model of HCMV DNApol is shown in Figure 10. In each case, the amino acid changes responsible for drug-resistance are listed and these residues are represented as a cloud of dots in the 3D-structure. There are more mutations in the Palm domain of HSV-1 DNApol than the HCMV counterpart, while the 3'-5' exonuclease domain of HCMV contains more mutations than its HSV-1 homologue. These two mutational profiles may describe two different mechanisms of drug-resistance, one favoring less drug incorporation into the viral genome (HSV-1) and the other activating the proofreading activity of the DNApol to excise the incorporated nucleoside analogues (HCMV). Interestingly, most mutations in VZV DNApol (89.5%) are in the Palm and Finger domains and confer cross-resistance to FOS. Notably, VZV DNApol E512K faces the Palm and Finger domains and its impact on FOS-binding may be significant since it is linked to FOS<sup>R</sup>. In contrast, the HCMV DNApol bears 50% of mutations in the 3'-5' exonuclease domain and has fewer mutations linked to FOS<sup>R</sup>.



**Figure 7.** Structural environment differences between the human DNApol  $\alpha$  and HSV-1 UL30 DNApol. **(A)** Sequence alignment of the Finger domain from HSV-1 UL30 and human DNApol  $\alpha$ . Conserved residues important for polymerase activity are in red and residues that differ are in blue. The asterisks (\*) show conserved positions and single (.) or double (:) dots show residues with similar physico-chemical properties. **(B)** DNApol  $\alpha$  active site in complex with dCTP and dT, based on RB69 gp43 DNApol structure. **(C)** HSV-1 UL30 DNApol active site based on RB69 gp43 DNApol structure. **(D)** FOS and acyclo-GMP binding the active site of RB69 gp43 DNApol. Magnesium ions are green balls and water molecules are red balls. The black dashed lines show the polar interactions. All the pictures were generated using PyMol Delano software.



**Figure 8.** Amino acid changes in herpesviruses DNaPol structure. The locations of the amino acid changes are shown in red. The published structure of HSV-1 UL30 (pdb: 2GV9) was the model for these 3D-structures of the different Herpesviruses DNaPol.

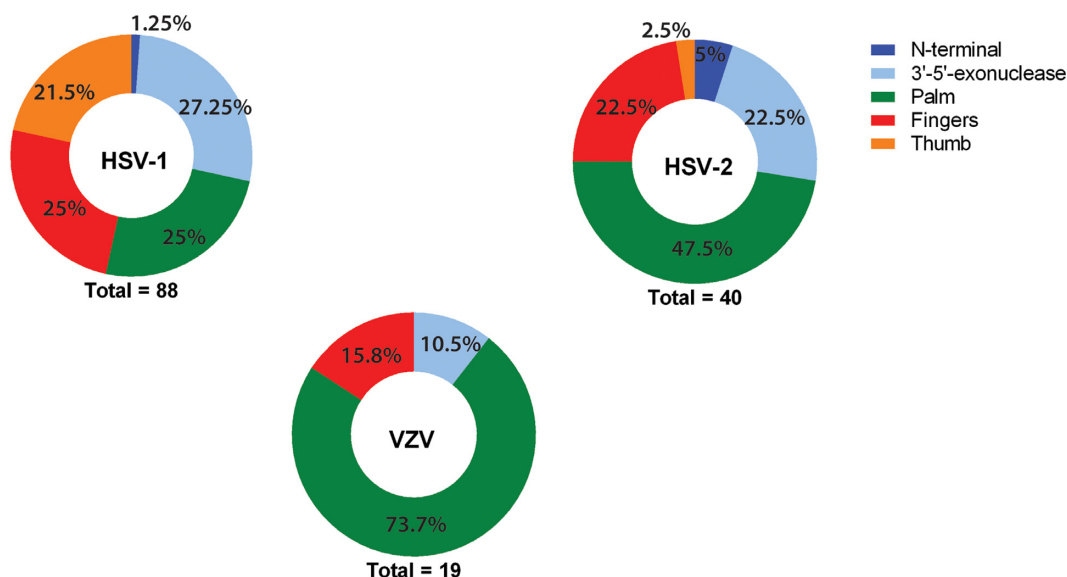
The  $\gamma$ -herpesviruses EBV and KSHV are oncogenic and therefore treatment of acute infections is only proposed in rare cases. Consequently, the emergence of drug-resistance mutations in EBV and KSHV has not yet been reported. Identification of mutations that could arise after long term exposure to anti-EBV and anti-KSHV agents may be important. Lytic induction therapy, an innovative approach for treating  $\gamma$ -herpesvirus-associated cancers, aims to reactivate EBV or KSHV from latency and then to inhibit lytic viral replication using nucleos(t)ide analogues (89–91). Surrogate models such as murine  $\gamma$ -herpesvirus 68 (MHV-68) and herpesvirus Saimiri (HVS) are useful to study drug-resistance in  $\gamma$ -herpesviruses (86). Resistance mutations in MHV68 and HVS were selected under drug pressure. In HVS, long term treatment with ACV selected two mutations, S529Y and L631I, in DNaPol Palm and Finger domains, respectively (86). These HVS mutant strains were cross-resistant to GCV, CDV, FOS and adefovir (a drug approved for treating hepatitis B virus infections that is also active against HSV-1 and other herpesviruses). The emergence of mutations conferring drug-resistance has to be considered, and therefore knowledge gathered from surrogate models such as MHV-68 and HVS would be crucial.

*Mutation distribution associated with nucleoside and pyrophosphate analogues.* Most mutations in HSV-1 DNaPol are associated with ACV<sup>R</sup>, yet some are associated with different profiles of drug resistance. Mutations

in DNaPol conferring resistance to ACV and GCV were identified that also confer cross-resistance to FOS or HPMP/PME derivatives. Amino acid changes S724N and I922N are associated with ACV<sup>R</sup>+FOS<sup>R</sup> (92–97). Interestingly, these amino acids are in the Palm domain, facing one another, within a 7 Å-pocket and are very close to the catalytic aspartates D717/D888 [S724 is 7 Å from D717 and 10 Å from D888, while I922 is equidistant (10 Å)]. Variations at positions V573M, R700M, K960R, L1007M and I1028T were found in HSV-1 clones selected with CDV (V573M, R700M and K960R) or HPMPA (W998L, L1007M and I1028T) in our laboratory. These positions are widely distributed in the Palm, Thumb and 3'-5' exonuclease domains (83,92). Although these mutations were selected under CDV or HPMPA pressure, there was no cross-resistance with ACV or FOS. The T821M, Y941H and R959H amino acid changes in the polymerase subunit (Finger/Palm/Thumb domains) are linked to ACV<sup>R</sup> and FOS<sup>R</sup> and to CDV sensitivity (83,93).

In HCMV UL54, amino acid changes N495K, D588E, T700A, V715M, E756D/N/Q and T838A confer only FOS<sup>R</sup>, and are widely distributed within the Palm, Finger and 3'-5' exonuclease domains. However, variations at positions 776, 781, 787, 802, 809 and 821 are exclusively in the Finger domain and confer cross-resistance to GCV and FOS. These observations suggest that variations linked to FOS<sup>R</sup> emerge preferentially in the Fin-



$\alpha$ -Herpesviruses $\beta$ -Herpesviruses

**Figure 9.** Distribution of the drug-resistance mutations in the *Herpesviridae* DNApol domains. The total number of mutations is shown for each virus.

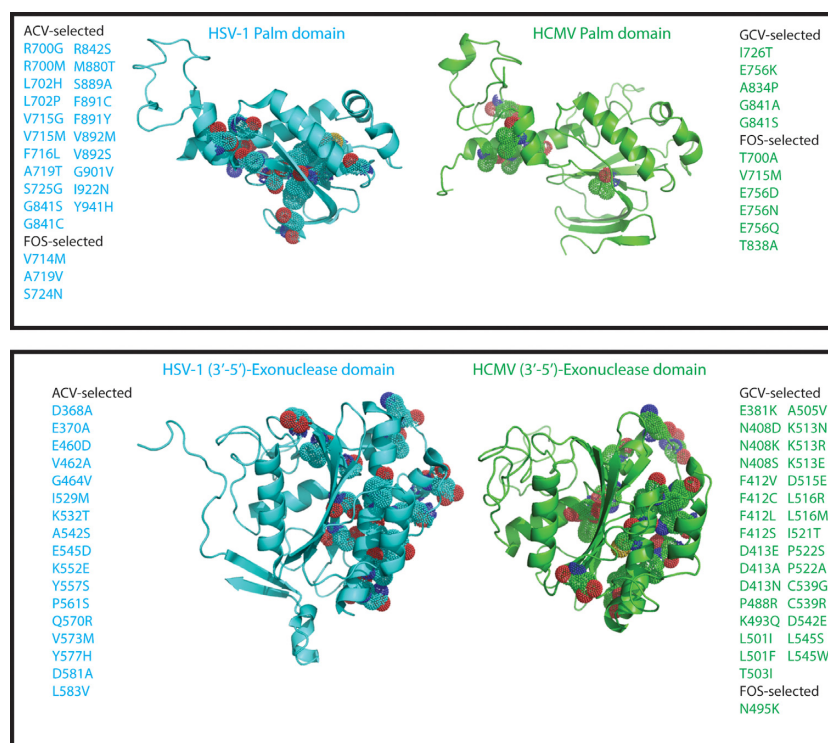
ger domain. Most GCV<sup>R</sup> mutations in UL54 also confer CDV<sup>R</sup>, and these are located in the 3'-5' exonuclease domain. Only one amino acid change in the Finger domain, K805Q, is exclusively associated with CDV<sup>R</sup>. Multidrug-resistance (GCV<sup>R</sup>+FOS<sup>R</sup>+CDV<sup>R</sup>) is associated with amino acid changes V812L, T813S, A834P, G841A and del981-982. Interestingly, these mutations are located in the Finger and Palm region of HCMV DNApol.

**Mutation distribution associated with HPMP and PME derivatives.** CDV (HPMPC) is the most potent of its class of antiherpesvirus agents, which are derivatives of (*S*)-1-[3-hydroxy-2-(phosphonylmethoxy)-propyl] (HPMP) and [9-(2-phosphonomethoxy)-ethyl] (PME). Mutations conferring resistance to CDV in HCMV have been isolated from patients mainly under GCV therapy, but more often were selected *in vitro* after long term exposure to the drugs. PME resistance mutations were isolated from patients in-

fectected with HSV-1, HSV-2, HCMV, HHV-6 under FOS therapy. Most drug-resistant VZV have been isolated in cultured cells (83,86,98–102). Figure 11 summarizes the mutation profiles in DNApol after selection with GCV or FOS (HCMV) and ACV or FOS (HSV, VZV), and whether these mutations confer cross-resistance to HPMP or PME derivatives.

In HSV-1 and VZV, mutations were identified in the Finger, Palm and/or Thumb domains (83,84,92). In HSV-1 DNApol, variations in residues S724N, E798K, L802F and R959H were selected under PMEA pressure (83,92), while Q618H, S724N, E798K and D1070N were identified in PMEDAP<sup>R</sup> HSV-1 clones (83,92). Cross-resistance to PMEA and PMEDAP was associated with these specific amino acid changes (Figure 11B). Interestingly, S724N arose using different antiherpesvirus agents (ACV, FOS, PMEA and PMEDAP), indicating a hotspot of genomic





**Figure 10.** Comparison of the Palm and 3'-5' exonuclease domains of HSV-1 and HCMV. The mutated residues are represented as dots in HSV-1 structural domains (cyan) and in the HCMV homolog domains (green). The amino acid changes were grouped according to the selection drug and listed by virus using the corresponding font color. Functional domains were visualized and the pictures generated using PyMol Delano software.

variability at this position. Mutations selected under CDV pressure, V573M, R700M and K960R, were in the 3'-5' Exonuclease, Palm and Thumb domains, respectively. These amino acid changes increased the CDV<sup>R</sup> fold-resistance from 5.6 to 14 and increased the sensitivity to FOS (hyper-sensitivity of 0.28- to 0.36-fold), while no cross-resistance was observed to PMEA and PMEDAP. However, these three mutations altered the sensitivity to HPMPA (12.5- to 26-fold) (83). Similarly to CDV, the amino acid changes selected under HPMPA treatment (W998L, L1007M and I1028T in the Thumb domain) conferred cross-resistance to CDV (83).

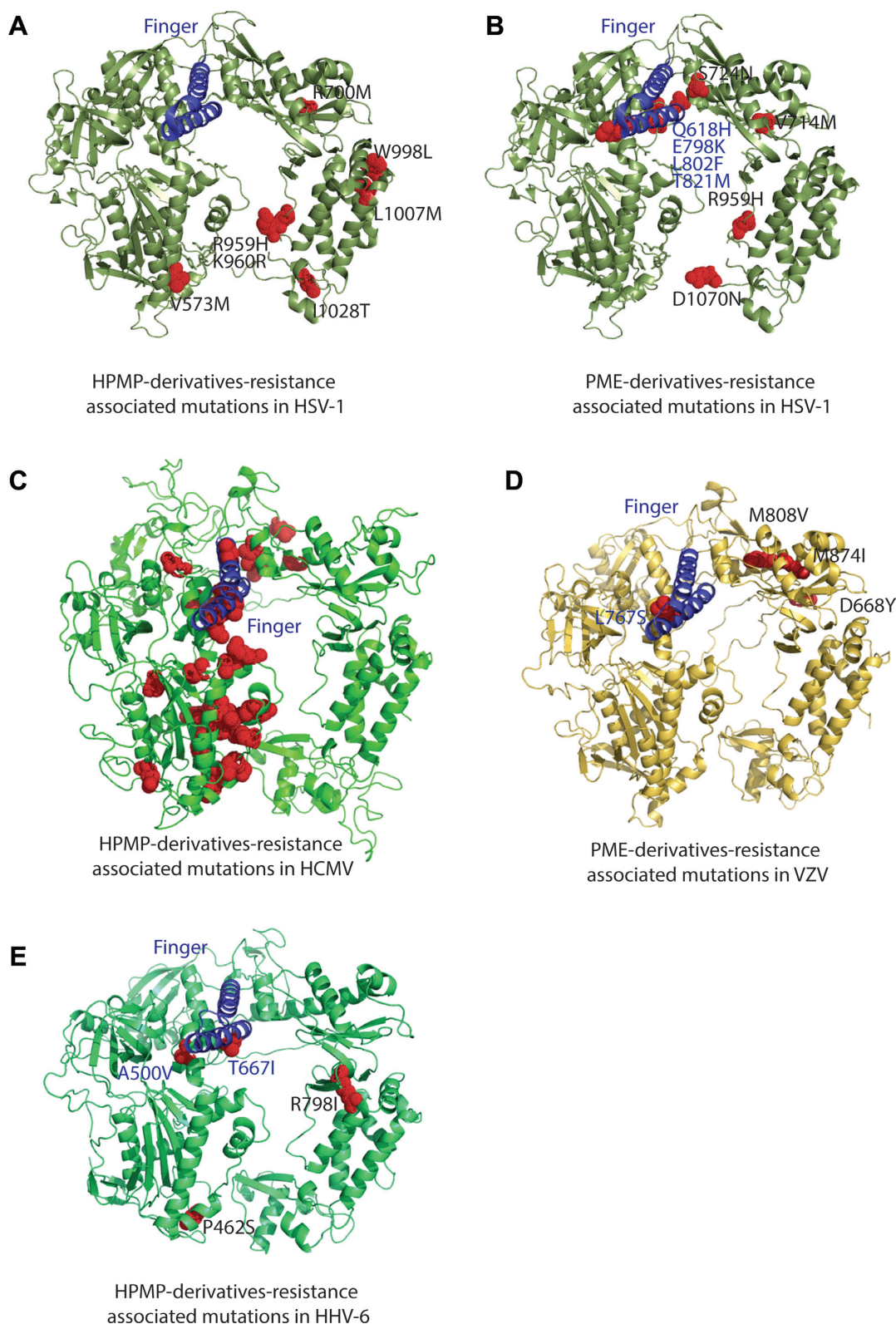
In VZV DNApol, L767S was selected under pressure with PMEA, and D668Y and M808V were selected under pressure with PMEDAP. L767S confers cross-resistance to PMEA, PMEDAP, ACV and PCV (Figure 11D). No amino acid changes in the N-terminus or the 3'-5'-exonuclease domains of HSV-1 and VZV DNApol arose under selection with PME derivatives. Interestingly, a distinct pattern of mutations in HSV-1 UL30 was associated with HPMP and PME derivatives. Mutations selected with HPMP derivatives were in the Palm, Thumb and 3'-5' exonuclease domains but not in the Finger domain, while mutations selected with PME derivatives were mostly in the Finger domain and some were in the Palm/Thumb domains (Figure 11A and B). In contrast to HSV-1 and VZV, more HCMV mutations conferring HPMP resistance were in the 3'-5' exonuclease and Finger domains (Figure 11C). A few mutations were also present in the Palm domain but none in

the Thumb domain. No mutations associated with PME derivatives in HCMV have been reported.

Most mutations in HHV-6 UL38 gene that confer drug-resistance were selected under FOS treatment (F292S, T435R, H507Y, C525S), and few were selected with CDV (P462S and R798I) (98,103,104). P462S maps to the 3'-5' exonuclease domain and R798I is in the Palm domain. Both P462S and R798I mutations confer cross-resistance to GCV, but only the homologous P462S substitution was also identified in GCV<sup>R</sup> HCMV (i.e. P522S). All four mutations selected under FOS pressure were in the 3'-5' exonuclease domain. T667I, detected in clinical samples, corresponds to the homologous position V812 in HCMV UL54, which confers cross-resistance to GCV, FOS and CDV. This position is located in the Finger domain of HHV-6 DNApol.

Taken together, these mutation profiles suggest different mechanisms of drug-resistance for HPMP and PME derivatives, with HCMV favoring the decrease of DNApol proof-reading activity (3'-5' exonuclease domain) while in HSV-1 the binding of the incoming nucleotide analogue is affected.

*Effects of drug-resistance amino acid changes on the kinetic parameters regarding antiherpesvirus agents.* The  $K_i$  values of recombinant, mutated viral DNApol were measured to study the effects of amino acid changes on the binding of antiherpesvirus drugs. These mutations were principally located in the NH2-terminus, Finger, Palm and Thumb domains. A series of ACV<sup>R</sup> mutations in HSV-1 UL30 were characterized by Huang *et al* (54). Three groups of amino acid changes were distinguished by their effects on  $K_M$  of



**Figure 11.** Mutations in the 3D-structure of herpesviruses that confer resistance to (A, C and E) HPMP-derivatives or (B and D) PME-derivatives. The mutations are represented as red dots and the Fingers domain is colored in blue. Panel C mutations: E381K, P401S, N408D/K/S, F412V/C/L/S, D413E/A/N/Y, V489I, K493Q, L501I/F, T503I, A505V, K513N/R/E, L516R/M, S517F, I521T, P522S/A, C539G/R, D542E, L545S/W, T552S, Q578H/L, I580T, D588E/N, I726T, E756D, L773V/F, S782L, K805Q, V812L, T813S, A834R and G841A/S.

ACV-TP, the catalytic constant  $k_{\text{cat}}$  of ACV-TP or dGTP, or both  $K_{\text{M}}$  and  $k_{\text{cat}}$ . E597K, R700G and R842S altered ACV-TP binding, changing the wild-type enzyme  $K_{\text{M}}$  from 0.81  $\mu\text{M}$  to 3.8, 5.1 and 3.1  $\mu\text{M}$ , respectively. These modifications increased ACV<sup>R</sup> 2- to 20-fold. These modifications did not affect the binding or the efficiency of incorporation of the natural substrate dGTP. R605V and F891C disturbed the ACV-TP incorporation rate, slowing the wild-type  $k_{\text{cat}}$  of 37  $\text{min}^{-1}$  to 0.61 or 1.8  $\text{min}^{-1}$ . These changes in  $k_{\text{cat}}$  increased ACV<sup>R</sup> 4- to 10-fold. S724N, Y941H and N961K altered both  $K_{\text{M}}$  and  $k_{\text{cat}}$  of ACV-TP. The apparent affinity  $K_{\text{M}}$  changed from 0.81  $\mu\text{M}$  to 2.7–4.7  $\mu\text{M}$ , and  $k_{\text{cat}}$  slowed from 37  $\text{min}^{-1}$  to 9.5–10  $\text{min}^{-1}$ . This caused an overall increase in ACV<sup>R</sup> of 4- to 7-fold. The mutations in this study are distributed in the NH2-terminus, Palm and Thumb domains of HSV-1 DNAPol.

Boivin *et al.* investigated the effects of W780V in HCMV UL54 and W781V in HSV-1 UL30, which confer resistance to FOS, GCV and/or ACV (76). In HSV-1 UL30, W781V increased the  $K_{\text{I}}$  from 0.04 to 1.8  $\mu\text{M}$  (45-fold), while in HCMV UL54, W780V increased the  $K_{\text{I}}$  from 0.55 to 2.7  $\mu\text{M}$  (4.9-fold).

Thesnokov *et al.* identified L802M (FOS<sup>R</sup>GCV<sup>R</sup>) and K805Q (CDV<sup>R</sup>) mutations in HCMV UL54 (78). An adjacent residue, Q807, was also mutated (Q807A). These amino acid changes affected the kinetic parameters of DNAPol toward the substrates. L802M and Q807A in the Finger domain increased the  $K_{\text{I}}$  of FOS from 0.30 to 1.9–2.0  $\mu\text{M}$ , leading to a fold-resistance of 6.3–6.7. Interestingly, K805Q conferred hypersensitivity to FOS, and the  $K_{\text{I}}$  decreased from 0.30 to 0.14  $\mu\text{M}$ . This amino acid change also conferred resistance to CDV.

Similarly, Cihlar *et al.* measured the inhibitory constants of GCV-TP, FOS and CDV-DP for HCMV DNAPol mutants K513N and V715M (62). Interestingly, K513N did not change the  $K_{\text{I}}$  for the tested compounds and there was no drug susceptibility correlation between HCMV and enzyme. However,  $K_{\text{I}}$  values increased for CDV-DP (2.4-fold), GCV-TP (2.9-fold) and FOS (12.8-fold) with V715M compared to wild-type enzyme. V715M altered drug binding and therefore a drug-sensitivity correlation is possible between HCMV and the DNAPol.

In contrast to mutations in polymerase activity domains, amino acid changes in the exonuclease domain may not alter GCV-TP incorporation. Coen *et al.* investigated the effects of D301N, F412V and L545S amino acid changes in the exonuclease domain on the kinetic parameters of HCMV UL54 DNAPol, regarding dGTP and GCV-TP (105). These amino acid variations had no effect on the affinity of the enzyme for GCV-TP. The  $K_{\text{I}}$  values of wild-type and mutant enzymes were 4.4–5.1  $\mu\text{M}$ . The efficiency of GCV-TP incorporation of the L545S mutant enzyme was slightly less ( $k_{\text{cat}} = 0.87 \text{ min}^{-1}$ ) than wild-type enzyme ( $k_{\text{cat}}$  of 1.7  $\text{min}^{-1}$ ).

In summary, mutations have multiple effects on viral DNAPol activity when antiherpesvirus agents are substrates. For instance, mutations modify the binding affinity ( $K_{\text{M}}$ ) of the nucleos(t)ide analogues or FOS, they impact the rate of incorporation ( $k_{\text{cat}}$ ) into the viral DNA or they alter both kinetic parameters. Interestingly, such changes might not be detectable when the mutations are located in the 3'-5'

exonuclease domain because the polymerization activity is not affected.

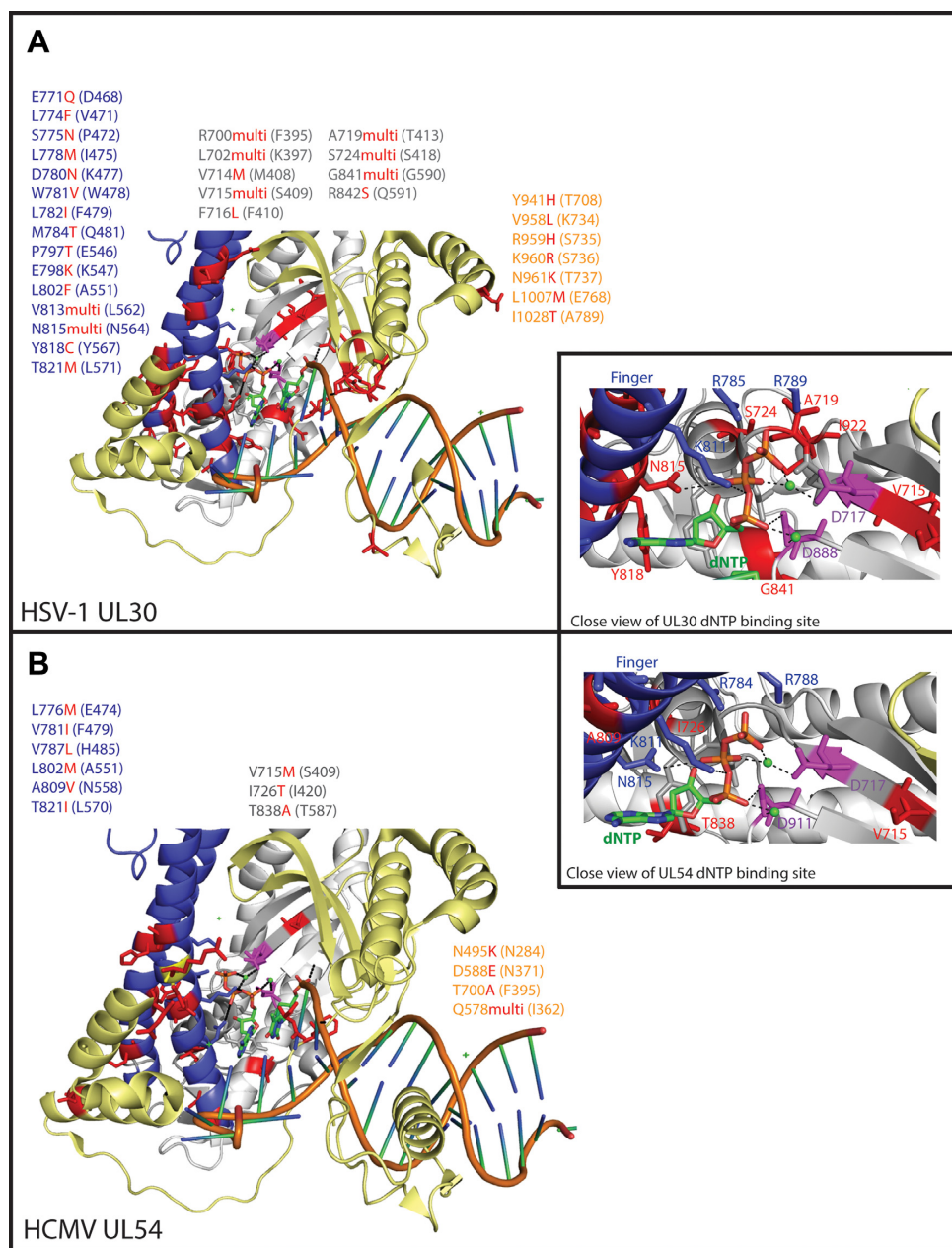
*Insights into binding alterations following acquisition of drug-resistance mutations.* The ACV mechanism of action is distinct from GCV, and this is reflected at the level of drug-resistance acquisition. ACV is a chain terminator, consequently drug-resistance mutations emerge in residues close to the  $\beta$ - and  $\gamma$ - phosphates or around the catalytic aspartates (D717/D888) to bypass the effects of its incorporation (Figure 12A). Amino acid changes at positions V714, V715, F716 and A719, which are close to D717, might alter the network of hydrogen-bonds with ACV-TP. In addition, alterations in R700, L702, G841, R842 and I922 influence the binding of ACV-TP, and these form a shell surrounding D717 (Figure 12A, close view). These amino acid changes exhibit broad biochemical characteristics, thereby affecting the catalytic aspartate and ACV-TP binding or incorporation.

Unlike the mutations observed in UL30 DNAPol, only three amino acid changes in UL54 DNAPol were identified in the shell of residues around the catalytic aspartate D717 (V715M, I726T and T838A). Six residues in the Finger domain and four residues that interact with the DNA strand were changed in UL54 (Figure 12B, inset). The mechanism of GCV resistance is likely different than ACV in drug-resistant HSV-1 strains (54). Coen *et al.* reported the effects of mutations D301N, F412V and L545S in the 3'-5' exonuclease domain (105). Their results suggested these mutations allow UL54 DNAPol to bypass GCV-TP incorporation and to continue DNA polymerization after  $n+1$  dNTP addition, leading to GCV in viral DNA strands. Foti *et al.* showed that an oligonucleotide duplex containing GCV has a modified three-dimensional structure compared to a natural oligonucleotide (71). Therefore, to continue viral DNA polymerization after GCV incorporation, amino acid changes are necessary to remodel DNA binding and impair exonuclease activity. This preferential drug-resistance mechanism might explain the high number of amino acid changes in the UL54 exonuclease domain, which is in contrast to drug-resistant HSV-1 strains that harbor mutations in the UL30 polymerase domain.

CDV incorporation by HCMV DNAPol does not alter the viral DNA three-dimensional structure (70). However, the rate of DNA elongation decreases dramatically, suggesting that DNAPol pauses in the presence of incorporated CDV. Two successive CDV incorporations induce DNA duplication arrest, and 3'-5' exonuclease activity cannot excise the incorporated CDV (48). The mechanism of CDV-resistance might involve defects in exonuclease activity to allow continuation of viral DNA elongation, possibly like the mechanism proposed for GCV (70,71). Most GCV<sup>R</sup>-mutations conferring cross-resistance to CDV are in the exonuclease domain. Position K805 was altered in HCMV UL54 and conferred resistance to CDV. Although K805 is in the Finger domain, it faces the 3'-5' exonuclease domain with a distance of 6.45 Å from residue K493 (K493Q found in GCV<sup>R</sup>+CDV<sup>R</sup>), based on the structure of RB69 gp43.

FOS binding is mediated through interaction with residues R784/K811 in HCMV UL54 and R785/K811 in HSV-1 UL30 in the Finger domain (Figure 13).





**Figure 12.** RB69 DnApol complexed to the DNA strand and the incoming dNTP. The amino acid changes (red) identified in (A) ACV/FOS and GCV/FOS drug-resistant HSV-1 and (B) HCMV. The homologous position in RB69 DnApol is in parentheses. The term ‘multi’ refers to a position that can be changed by different types of amino acids. The catalytic aspartates are shown in magenta. The Finger domain is composed of blue helices, while the Palm domain is shown in grey and yellow. Insets focus on the incoming dNTP binding to UL30 and UL54 DnApol.

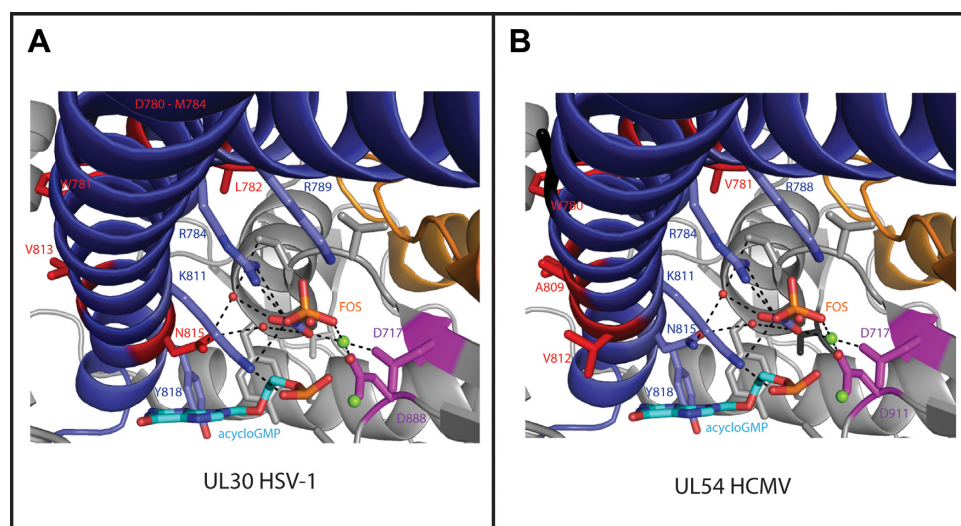
These specific positions are not associated with mutations, but changes in the surrounding positions were identified [M784T, L782I, W781V, D780N, V813M/A, N815L/V/Y/E/S/Q/T in HSV-1 UL30 (Figure 13A) and W780V, V781I, V787L, L802M, A809V, V812L and T813S in HCMV UL54 (Figure 13B)]. Surprisingly, amino acid changes in the 3′-5′ exonuclease or Palm domains, outside the Finger domain, conferred FOS-resistance (N495K, D588E, T700A, V715M, E756D/N and T838A). The mutations might modify the catalytic aspartates or interactions with the single-strand DNA, allowing new dNTP incorpo-

ration and elongation. Alternatively, distal mutations might alter the distance between FOS and the interacting residues, thereby reducing its binding affinity.

## NEW DEVELOPMENTS IN ANTIHERPESVIRUS TREATMENT: WHAT’S THE NEXT STEP?

Several compounds at different stages of drug development are currently being tested for safety and efficacy against herpesvirus infections (Figure 14). These compounds are structurally related or unrelated and target different viral proteins. New strategies involving cellular proteins that are hi-





**Figure 13.** Magnified view of the (A) HSV-1 and (B) HCMV DNApol active sites in complex with FOS and acyclo-GMP, based on the three-dimensional structure of RB69 gp43. Amino acid changes identified in FOS-resistant strains are shown in red.

jacked by the virus are also under investigation. Inhibition of cellular proteins, including disrupting interactions with viral proteins or inhibiting enzymatic activity, might be effective against herpesviruses. In this section, new drugs and target candidates are described, including those in clinical trials.

### Nucleotidic inhibitors of viral DNA polymerase

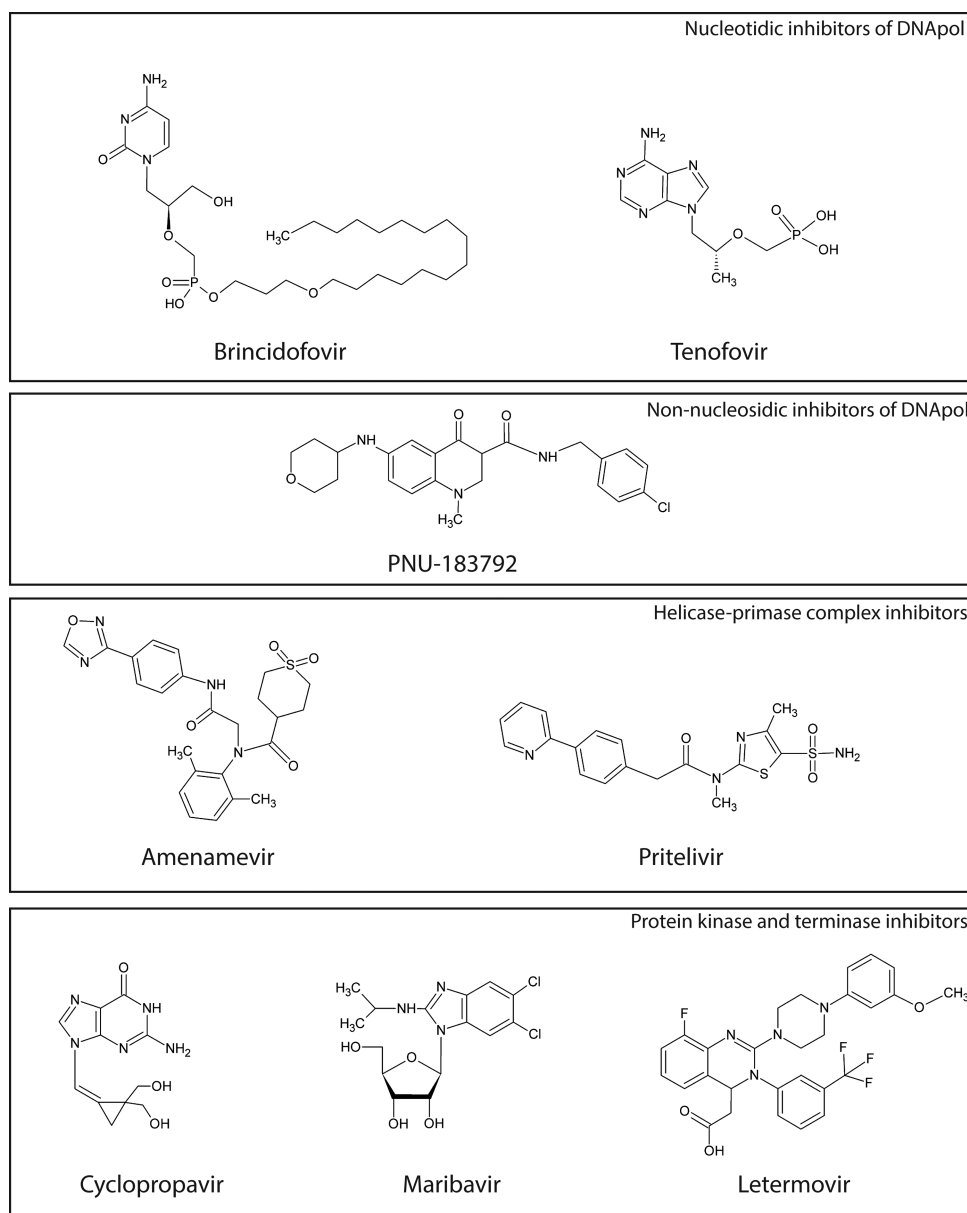
**Brincidofovir.** Brincidofovir (BCV, CMX-001) is a pro-drug of cidofovir that is effective against several DNA viruses. Clinical trials are underway to test its efficacy against several human herpesviruses (106–112). Chimerix, Inc. conducted a Phase II trial in HCMV seropositive hematopoietic stem cell transplant recipients to assess BCV safety, tolerability and activity against HCMV (NCT00942305). This study was completed in January 2012 and demonstrated efficacy of oral BCV against HCMV at 100 mg twice weekly, with gastrointestinal toxicity at 200 mg twice weekly (110). Two Phase III studies started in May 2015 in HCMV seronegative (NCT02439970) and seropositive (NCT02439957) kidney allograft recipients to compare BCV efficacy versus val-GCV. These clinical trials aim to assess prevention or control of HCMV infection in transplant patients. These programs are active but not yet recruiting patients. BCV is an interesting compound that will avoid CDV nephrotoxicity since it displays a lack of OAT1-dependant excretion in the urine (113). However, its use will be limited to CDV-sensitive herpesvirus strains. It was recently reported that BCV resistance mutations in HCMV UL54 were selected *in vitro* (114). Three are in the 3'-5' exonuclease domain (E303G/D, N408K, D413Y) and one is in the Finger domain (V812L).

**Tenofovir.** Tenofovir (TFV, PMPA) is a dAMP nucleotide analogue with activity against HIV and HBV (115). Its mechanism of action requires phosphorylation by cellular kinases and incorporation in the viral genome by viral polymerase (116–118). In its active diphosphate form, tenofovir-

DP inhibits HSV-1 DNApol with an  $IC_{50}$  of 0.38  $\mu$ g/ml in the presence of competing dATP (at 3.2  $\mu$ M) (119). A TFV 1% intravaginal gel is also active against HSV-1 and HSV-2 infections (119, 120). Clinical trials were completed to assess the efficacy of TFV in preventing herpes simplex infection. The effect of TFV on genital herpes simplex virus (HSV-2) shedding was assessed in HIV seronegative women in a Phase IV study (NCT01448616) (121). Intravaginal TFV gel efficacy was compared to a placebo vaginal gel, oral TFV disoproxil fumarate (TDF, Viread®) and oral placebo. Oral TDF failed to prevent lesions or shedding of HSV-2, whereas vaginal TFV gel reduced HSV-2 shedding by 60%. A Phase III double blind multi-center study was completed regarding the safety and efficacy of TFV 1% intravaginal gel in preventing HIV-1 and HSV-2 infection in sexually active women (NCT01386294). Some protection against HSV-2 infection in HSV-2 seronegative women was observed upon pericoital use of TFV 1% intravaginal gel (122). However, more potent topical antiherpesvirus agents based on ACV or CDV should be designed and tested in combination with anti-HIV drugs in a microbicide formulation.

### Non-nucleosidic inhibitors of viral DNA polymerase

The use of non-nucleosidic inhibitors of viral DNApol is an option to address multidrug-resistant infections. Pharmacia Corp. used high throughput screening to identify a new class of non-nucleosidic inhibitors of herpesvirus replication (123,124). One of the most promising compounds, 4-oxo-dihydroquinoline-3-carboxamide (PNU-183792), was effective against HCMV, HSV-1, HSV-2, VZV, KSHV and EBV (when compared to ACV or GCV as reference drugs) (32,125–128). Liu *et al.* proposed a model for PNU-183792 activity against HSV-1 that involves stacking the drug between the primer 3'-end and the template base pairs (32). However, Q618H in HSV-1 DNApol was selected with PMEDAP and conferred resistance to PNU-183792 (83). This demonstrates the difficulty of discovering new an-



**Figure 14.** Chemical structure of newly tested compounds with antiherpesvirus activity.

tiherpesvirus drugs because different chemical classes of compounds might not be effective against multi-drug resistant strains if they target the same viral protein. Although promising, PNU-183792 has not been developed as an antiherpesvirus drug.

#### Antiherpesvirus inhibitors under validation process

**Helicase–primase inhibitors.** The helicase–primase complex required for replication of the viral genome is a potential target of high interest. Two molecules, amenamevir (ASP2151) and pritelivir (AIC316; BAY-57-1293), have undergone in Phase III and Phase II (NCT01047540) clinical trials, respectively (129).

Amenamevir was initially developed by Astellas Pharma Inc. for treating drug-resistant HSV-1 and VZV infections

(130–134). Its development was halted in 2010 due to toxicity observed during a Phase I study (NCT00870441). The results of this Phase I study have not been reported. Two Phase II studies regarding the effects of amenamevir on herpes zoster and herpes *genitalis* were performed in Japan (NCT00487682, NCT00486200). One-day or 3-day treatments with amenamevir were effective against episodes of recurrent genital herpes (135). In 2013, Maruho Co. Ltd. continued the development of amenamevir and registered a double-blind and placebo-controlled Phase III trial for HSV and VZV (NCT01959295, NCT01959841, NCT02209324). However, patient recruitment has stopped and no results have been published.

Pritelivir was developed by AiCuris and brought to a Phase II trial in patients with genital HSV-2 infection (NCT01047540) (136–138). Some toxicological concerns

suspended drug development, mainly dermal and hematological issues in monkeys receiving high doses of pritelivir. However, this study showed a dose-dependent reduction in HSV-2 shedding and days with lesions (129, 137). Amenamevir and pritelivir have good antiviral activity against HSV-1 and HSV-2, but toxicity must be overcome for them to be suitable antiherpesvirus drugs. Less toxic analogues of these helicase–primase complex inhibitors might unlock the route for new antiherpesvirus agents that would facilitate the management of herpesvirus-associated disease, including infections caused by drug-resistant strains.

**Protein kinase and terminase inhibitors.** UL97 protein kinase and UL89 terminase in HCMV are newly identified targets (139–146). Maribavir and cyclopropavir (CPV) inhibit HCMV UL97 protein kinase (147). CPV is also activated by UL97 protein kinase and cellular GMP kinase to inhibit HCMV DNApol (148–151). Therefore, CPV has a dual mechanism of action against HCMV (148,152). HCMV terminase inhibitors are letermovir (MK-8228; AIC246), BAY-38-4766, 2,5,6-trichloro-1- $\beta$ -D-ribofuranosyl benzimidazole (TCRB) and 2-bromo-5,6-dichloro-1- $\beta$ -D-ribofuranosyl benzimidazole (BDCRB). Letermovir development is currently ongoing. A Phase II study was completed, showing its safety and efficacy against cytomegalovirus reactivation in bone marrow transplanted patients, with an optimal dose at 240 mg per day (NCT01063829) (153). A Phase III trial is currently recruiting patients (NCT02137772).

**Processivity factors binding inhibitors.** Accessory proteins required for herpesvirus DNA replication and interactions with DNApol might also be targeted. Processivity subunits UL42 (HSV-1) and UL44 (HCMV) are associated with the UL30 and UL54 DNApol, respectively, through protein–protein interactions with the C-termini (154–157). In 2004, a study showed that it was possible to inhibit interactions between HSV-1 UL30 and UL42 proteins with a small molecule, BP5 (158). Similarly, BP5 inhibited association between feline herpesvirus DNApol catalytic subunit and its processivity subunit (159). To our knowledge, BP5 is not under development. It was reported that raltegravir, an HIV integrase inhibitor, was also active against HSV-1 by interfering between UL42 and UL30 proteins. A raltegravir-resistant HSV-1 clone possessed a mutation in the UL42 gene (V296I) (160). This amino acid change was in the UL42 C-terminal processivity subunit, close to the interaction interface that binds to UL30 DNApol. Raltegravir is also active against HCMV and HSV-2 (160).

### Next generation antiherpesvirus inhibitors: which target?

**Viral proteins.** Proteins associated with viral DNA replication can be good alternative targets to circumvent multidrug-resistant strains. Several viral proteins promote synthesis of NTPs required for viral DNA replication. The uracil-DNA glycosylase excises uracil residues after misincorporation of dUMP by viral DNApol. It is especially important in terminally differentiated neurons because DNA repair is attenuated compared to undifferentiated dividing-cells (161–164). Its three-dimensional structure was solved and could be used to design inhibitors

*in silico* (165). Another enzyme associated with dUMP metabolism is the dUTP nucleotidohydrolase, which is required to reduce dUTP misincorporation into viral DNA. Its inhibition might impair viral replication (166–168). The ribonucleoside–diphosphate reductase converts ribo-NDP to deoxy-NDP, and it is involved in viral growth in non-dividing cells and reactivation from latency. Several studies qualified this enzyme as a possible target for herpesvirus replication inhibition (169–171). VZV ORF13 thymidylate synthase (TS) was recently characterized, and its three-dimensional structure was solved by Nordlund *et al.* (pdb code: 4XSE, 4XSD and 4XSC) (172). They determined that BVDU monophosphate (BVDU-MP) is a ligand of VZV TS and suggested rational modification of compounds like BVDU-MP could have greater affinity for the active site. The physiological relevance of VZV TS as a therapeutic target needs further investigation.

The three-dimensional structures of several viral proteases were solved and can be used to design inhibitors *in silico*: HCMV UL80 (pdb code: 1WPO and 1IEG) (173–175), VZV ORF33 (pdb code: 1VZV) (176), HSV-2 UL26 (pdb code: 1AT3) (177), KSHV ORF17 (pdb code: 1FL1, 2PBK, 3NJQ, 4P2T, 4P3T) (178–180) and EBV BVRF2 (pdb code: 1O6E) (181).

**Cellular targets.** Cellular partners of the herpesvirus life cycle that could be investigated include those involved in virus entry, gene transcription, viral protein maturation, capsid assembly and maturation or virion egress. Mues *et al.* evaluated dynasore, a small-molecule inhibitor of dynamin, on HSV-1 and HSV-2 life cycle. Dynasore affected virus entry, trafficking of viral proteins and capsid formation of HSV-1 and HSV-2 (182). Cheshenko *et al.* identified a link between Akt and calcium signaling induced by HSV-1 that promote virus entry (183). Miltefosine, an anti-leishmania drug that blocks Akt phosphorylation, was active *in vitro* against ACV<sup>R</sup> HSV-1 by inhibiting HSV-induced calcium release and virus entry.

### Therapeutic vaccines against herpesvirus infection

Therapeutic vaccines are an alternative strategy to the use of antiviral agents. Two such vaccine candidates are currently in clinical trials for the treatment of genital herpes, HerpV (Agenus<sup>®</sup>) and GEN-003 (Genocoe Bioscience<sup>®</sup>) (184–186). A Phase II clinical trial assessing the efficacy of HerpV (NCT01687595) showed that a synthetic vaccine composed of HSV-2 antigenic peptides caused a significant decrease in viral load (75%). A booster shot given six months after the first vaccination was compatible with sustained immunity. A Phase II clinical trial assessing the efficacy of GEN-003 is ongoing and results are expected by late 2016 (NCT01667341). GEN-003 showed promising results during a Phase II dose optimization study. HSV-2 shedding (64%) and lesions (65%) were reduced 12 months after vaccine completion. No booster shot was tested during the optimization process. These therapeutic vaccines had effects on viral replication and on the clinical aspects of the diseases, with significant reduction of genital lesions. They are expected to contribute to the management of HSV-2 infections.



## CONCLUSIONS AND PERSPECTIVES

A distinct mechanism of drug-resistance adopted by HSV-1, HSV-2 and VZV ( $\alpha$ -herpesviruses) compared to HCMV and HHV-6 ( $\beta$ -herpesviruses) was determined by analysis of DNApol mutants and generation of a distribution profile. Insight into the mechanism of drug-resistance is an asset in developing new antiviral agents that target the herpesvirus DNApol. Accounting for amino acid changes responsible for drug-resistance provides the basis for new scaffolds or different chemical modifications.

Since the discovery of nucleoside analogues ACV and GCV, antiviral research for herpesviruses made a step forward by the design of molecules (i.e. FOS and CDV) that bypass resistance due to alteration in the viral TK or PK. Viral TK (encoded by HSV-1, HSV-2 and VZV) and PK (encoded by HCMV and HHV-6) are required to activate ACV and GCV, respectively, and so mutations in these genes generated ACV- or GCV-resistant strains. The pyrophosphate analogue FOS targets DNApol directly without an activation step, while CDV has a phosphonate moiety that does not require TK or PK activation. However, CDV<sup>R</sup>- (selected under GCV therapy) and FOS<sup>R</sup>-HCMV strains emerged in immunosuppressed populations over the last decade, reducing the therapeutic options (27,187–189). Mutations selected under GCV pressure, for example, led to cross-resistance to CDV and/or FOS. The consequences are life-threatening HCMV infections and lack of effective treatments for managing HCMV-associated diseases. Therefore, new strategies are needed and new targets must be discovered to face the health problems caused by drug-resistant herpesviruses.

The long term success of new nucleoside/nucleotide analogues, or any molecules that target herpesvirus DNApol, might be diminished due to existing or new mutations in TK, PK and DNApol. Promising therapeutic strategies for managing infections with multi-drug resistant strains are currently under investigation. Development of therapeutic vaccines to confer protection against HSV-1/HSV-2 and decrease herpes episodes is also worth effort. An anti-HCMV vaccine could also be developed for populations suffering from particular infections such as congenital CMV and CMV disease in transplant recipients. Several HCMV vaccine studies have been initiated and clinical trials performed, but results were unsatisfactory or the investigations are still ongoing (190–193).

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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